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AN INTRODUCTION
TO
BACTERIOLOGICAL AND ENZYME CHEMISTRY

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AN INTRODUCTION
TO
BACTERIOLOGICAL
AND
ENZYME CHEMISTRY

BY

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TO

A. H. F.

PREFACE

THE subject of bacteriological and enzyme chemistry is becoming year by year of increasing importance. A knowledge of it is now necessary for the scientific conduct of many industrial processes of great magnitude.

Apart from its well-known applications in the fermentation industries, a scientific understanding of this branch of chemistry is likely to exercise considerable influence upon the future development of agriculture.

Recent advances in sanitation, especially the provision of pure water, and the inoffensive disposal of sewage, call for the co-operation of the engineer and the biological chemist.

The Institute of Chemistry has recognised these requirements in the special examination in biological chemistry, which it has conducted for a number of years past. The author's students have frequently asked him to recommend an elementary book, which should serve as an introduction to the somewhat overwhelming literature of the subject.

The difficulty of pointing to any one work which satisfied these requirements led the author to attempt himself to supply the deficiency.

In writing the book he has had in mind, not only the purely chemical student, but also members of other professions, with whom he has frequent occasion to co-operate, notably the engineer and medical officer of health, as well as the general reader, to whom the subject offers many attractions.

On this account certain chapters of the book especially, e.g., those on the principles of organic chemistry, have been written in a more elementary manner than would be called for by the pure chemist. On the other hand, the methods of experiment and research employed in bacteriological and enzyme chemistry have been illustrated in some detail by typical examples.

The endeavour has also been made to keep the style interesting and readable, without sacrificing scientific accuracy, How far this object has been attained it will be for the reader to judge.

It is impossible for any one scientific worker to be a specialist in more than, at most, a very few branches of study. The author has been fortunate in obtaining valuable assistance in the writing of this book from many of his scientific colleagues. In particular he would gratefully acknowledge the help which he has received from Professor Adrian Brown of the University of Birmingham, Dr. A. Harden of the Lister Institute, Dr. E. J. Russell of the Rothamsted Experimental Station, Dr. H. H. Mann, Chemist to the Indian Tea Association, and Mr. S. H. Davies, Chemist to Messrs. Rowntree and Co.

Other references will be found in the body of the book, or in the short bibliography at the end. The latter, while comprising only important text-books, and original papers of fundamental interest, will, it is hoped, enable the student to continue his reading, and to follow up the subject in any direction, by means of the fuller bibliographies in the works cited.

In conclusion it is only right to mention the great assistance the author has received from his wife, who has acted as his amanuensis, and to whom this book is dedicated.

G. J. F.

January, 1911.

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AN INTRODUCTION

TO

BACTERIOLOGICAL AND ENZYME CHEMISTRY

CHAPTER I

THE CHARACTERISTICS OF CHEMICAL ACTION IN LIVING MATTER

The student of chemistry must always be impressed with the extraordinary ease with which complicated chemical changes take place in living matter. By comparison the methods used in the laboratory to effect the artificial preparation of natural products appear cumbersome and violent.

Thus, e.g., to take a fairly simple case, the colouring matter alizarine is produced in the madder plant under natural conditions of growth; at temperatures, that is, much below the boiling-point of water and without the production of any excessive alkalinity or acidity.

To prepare this substance artificially a hydrocarbon anthracene is made use of, itself produced by the distillation of coal tar at a high temperature. This is first violently oxidised by reagents such as bichromate of potash and glacial acetic acid; the resulting oxidised product anthraquinone is then dissolved in concentrated acid, the sulphonic acid so obtained

converted into a lime salt by the addition of lime, and the lime salt finally fused with caustic soda, producing the sodium salt of di-hydroxy-anthraquinone or alizarine.

The artificial preparation of such substances as indigo, camphor and terpenes, uric acid, etc., is even more complicated, although the actual chemical reactions may not always be of so drastic a character.

The same contrast between natural and artificial processes is observable when the change results in the decomposition of substances. Thus to saponify a fat, i.e., to split it up into its constituents, viz., a fatty acid and glycerine, by chemical means, high pressure steam or strong acid or alkali is necessary, a condition of things which obviously does not obtain in the ordinary processes of fat digestion in the body. Moreover certain chemical changes which have so far not been artificially produced are brought about with the greatest ease by living matter ; thus, e.g., cellulose, a carbohydrate of the general formula $(C_6H_{10}O_5)_n$ can be split up by fermentation into marsh gas, CH_4, hydrogen, H, and carbon dioxide, CO_2, and various subsidiary products. This change can be observed in nearly any green stagnant pond, the mud on the bottom of which generally yields copious bubbles of gas if stirred, and one of the famous frescoes by Ford Madox Brown in the Manchester Town Hall represents John Dalton, Manchester's great chemical philosopher, collecting marsh gas in this way.

The well-known and extremely important alcoholic fermentation of grape sugar is similarly instructive. By the action of yeast this readily yields alcohol and carbon dioxide roughly in accordance with the following equation :—

$$C_6H_{12}O_6 = 2C_2H_5OH + 2CO_2$$

In this case also, simple as the change appears, it has not been hitherto possible to bring it about under strictly artificial conditions.

In seeking to elucidate the conditions under which these

chemical changes take place in nature they may be compared in the first place with ordinary chemical changes, which can be effected in the laboratory with a minimum of assistance from external chemical or physical energy. Examples of such changes are frequent in the category of so-called *catalytic actions.*

The little cigar lighter, a smoker's toy which is often to be seen in tobacconists' shops, is a good illustration of the chemical action brought about by *catalysis.* In this case the warmth of the hand causes a little alcohol vapour to evaporate from the metal box and to impinge on a small knob of spongy platinum which acts as the catalyst. Its precise mode of action is not fully known, but it has the effect of bringing about the union of the alcohol vapour with the oxygen of the air, with the result that the alcohol bursts into flame. Platinum in a state of fine division, such as may be obtained, for example, by soaking asbestos in platinum chloride and driving off the chlorine by heat, is capable of bringing about a number of changes at temperatures much below those at which they would normally take place, and in some cases these changes are such as would not otherwise occur. If a thread of asbestos, covered with platinum in the manner above described, is warmed and then held in a stream of coal-gas escaping, e.g., from an unlit Bunsen burner, the platinised asbestos will glow.

A technical process of importance, viz., the manufacture of highly concentrated sulphuric acid, consists in passing sulphur dioxide (SO_2), obtained by burning pyrites or sulphur, together with oxygen, or air, over heated platinum in a fine state of division. The two gases then combine in accordance with the simple equation :—

$$SO_2 + O = SO_3$$

But this change does not take place directly, i.e., without the presence of a substance like the platinum, which acts as a catalyst.

In the case of spongy platinum and other finely divided metals the chemical change is accelerated in a large degree by *physical* causes ; a finely divided metal presents an extended surface on which the reacting substances are brought into intimate union. Chemical agencies may be at work at the same time, e.g., the formation of unstable intermediate compounds such as oxides or hydrides ; but the physical conditions are probably the governing factor.

It is otherwise with certain other catalytic changes, notably, e.g., the combination of sulphur dioxide with oxygen through the intervention of nitric oxide, which is the basis of the chamber process for the manufacture of sulphuric acid. Sulphur dioxide does not combine directly with oxygen, but when oxygen is presented to it in combination as nitrogen peroxide, it is easily oxidised with simultaneous formation of nitric oxide. Nitric oxide, on the other hand, readily combines with the oxygen of the air, again producing nitrogen peroxide. The changes are expressed in the following equations :—

$$NO + O = NO_2$$
$$SO_2 + NO_2 = SO_3 + NO$$

It will thus be seen that in presence of oxygen, or of course of air, a very small amount of nitric oxide (NO) is capable of converting an indefinite quantity of SO_2 into SO_3, itself remaining unchanged at the end of the process.

On the large scale this change takes place in the vast leaden chambers which cannot fail to be noticed in centres of chemical industry, such as Widnes. The various gases are introduced into these chambers, together with steam. The steam, H_2O, and SO_3 together form sulphuric acid, H_2SO_4, which collects on the floor of the chamber.

The catalytic action of nitrous fumes can be readily shown in the laboratory by shaking a solution of ferrous sulphate (green copperas) with a little nitrite of soda and sulphuric acid in a bottle nine-tenths full of air. The green colour

of the copperas solution quickly changes to yellow, owing to the formation of ferric sulphate, according to the following equation :—

$$2FeSO_4 + H_2SO_4 + NO_2 = Fe_2(SO_4)_3 + NO + H_2O$$

The NO combines with the oxygen in the air present to form NO_2 and so continues the reaction. This process has been made the subject of a patent, and is used to prepare ferric salts on the large scale for the purpose of precipitating sewage.

Another important case of catalytic action is the action of manganese dioxide on the decomposition of potassium chlorate by heat ; the temperature at which oxygen is evolved from potassium chlorate on heating is very much reduced by the addition of a comparatively small amount of manganese dioxide. In this case also it has been shown by McLeod, the present writer and others, that the action of the manganese dioxide is probably due to the formation and decomposition of intermediate substances.

The reactions which take place in living matter come, in many cases, under the order of catalytic reactions. The nature of the catalyst is one of the problems for consideration. These catalysts occurring in living matter are known as *enzymes* or ferments, and their varying effects form the chief subject-matter of this book.

Many of the reactions which take place in nature can be imitated in the laboratory by fairly simple methods ; thus, e.g., cane sugar is easily converted into grape sugar by warming for some time with dilute acid, according to the following equation :—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Ethereal salts or esters of the simpler fatty acids, such as, e.g., ethyl acetate, can be broken up by warming with dilute acid or alkali, yielding alcohol and acetic acid. Such a reaction does not proceed to completeness under ordinary

conditions, but ceases when a certain definite proportion of the ester has been broken up. Such a reaction is known as a *reversible reaction*, and is generally written thus :—

$$CH_3COOC_2H_5 + H_2O \rightleftarrows C_2H_5OH + CH_3COOH$$

The changes above described are typical of a series of reactions characterised by the absorption of the elements of water ; such a process is generally referred to as *hydrolysis*.

A great many fermentative changes are hydrolytic in their character and consequently of a very simple order. It was at one time considered that under natural conditions only changes took place which were essentially of this order, and in which there was always a liberation of heat as a result. Recent research has, however, shown that this generalisation does not hold, it being possible to build up substances by the action of enzymes, as well as to break them down. It is probably more correct to say that enzyme actions are, strictly speaking, reversible, but that the reaction takes place in both directions only under special conditions.

Besides the multiplicity of *chemical* agents already mentioned, the chemist has at his disposal means for varying at will within wide limits the *physical* conditions of reaction.

Temperature and concentration have already been mentioned, but it is also possible to remove one or more of the reacting bodies from the sphere of action by distillation, either at the ordinary or at reduced pressure. Filtration through various kinds of filtering media is possible, or separation by varying solubilities.

Under natural conditions the choice of methods is obviously much more restricted, and therefore before going farther it will be well to consider more closely the conditions under which chemical actions actually do take place in nature, and for this purpose to devote some attention to what may be termed nature's ultimate laboratory, that is, a living cell.

The unit of all living matter is the *cell*. Broadly speaking,

the cell consists of an envelope which can be described as semi-permeable, that is, permeable to one class of bodies but not to another. The contents of the envelope consist of liquid plasma or sap, throughout which, and lining the interior of the envelope, is a semi-fluid mucilaginous substance referred to generally as *protoplasm*. This is in a continual state of movement and of chemical change; and in the midst of it is a cell nucleus.



Fig. 1.—Typical Living Cell.

The substances entering or leaving the cell must obviously be possessed of certain physical properties if they are to pass through the semi-permeable membrane. It is necessary, therefore, to consider the different conditions which the matter composing the various substances entering and leaving the cell laboratory may assume. There are first solid insoluble bodies; these, of course, are not likely to pass through the cell membrane; on the other hand, substances in true solution, such as, e.g., salt dissolved in water, will as a rule pass freely through. There are, however, intermediate conditions in which matter can exist.

Colloids.—It was first shown by Graham that by appropriate means solutions could be obtained, which, while devoid of visible particles, were incapable of passing unchanged through a parchment membrane. Substances which were soluble and which would pass while in solution through a parchment membrane Graham termed *crystalloids*; substances which while soluble as judged by ordinary physical tests would yet not pass through a parchment membrane he termed *colloids*. A typical case illustrating the difference between a colloid and a crystalloid is the one selected by Graham, viz., silicate of soda. If a dilute solution of silicate of soda is carefully acidified with hydrochloric acid, no precipitation

takes place; if the solution is now placed in a cylindrical vessel one end of which is closed by a parchment diaphragm and the whole immersed in clean water, which is renewed from time to time, the sodium chloride formed by the action of the hydrochloric acid on the sodium silicate will diffuse through the parchment and eventually be completely removed. The silicic acid will remain behind in the cylinder. The sodium chloride in this case is the crystalloid, the silicic acid the colloid. The apparatus used in the experiment is known as a dialyser and the process as *dialysis*.

A large amount of work has been done of recent years on the chemistry of colloids. It has been shown that no very marked line can be drawn between the two extremes of matter in the solid insoluble condition and matter in true and crystalloid solution. The following are, however, typical properties of colloids :—

(1) When examined by an instrument known as the **ultramicroscope,** colloidal solutions are all found to contain particulate matter, that is, matter in an extremely divided state but still existing as separate particles.

The ultramicroscope is an instrument whose design is founded upon what is known as the ' Tyndall phenomenon.' The lighting up by a sunbeam of the dust in the atmosphere of a room is a matter of common observation. Tyndall found that if a closed space was rendered ' optically empty ' by smearing its sides with glycerine and allowing all particles to subside and be caught by the glycerine, a beam of light on passing through was invisible when viewed at right angles to its path. On admitting a little smoke the path of the beam at once became visible. The same phenomenon is observed with solutions. A solution perfectly free from suspended particles allows a beam of light to pass through and remain invisible. On introducing a colloid substance such as gum-mastic into the solution the path of the beam at once lights up.

The application of this phenomenon to the study of

colloidal matter has been the subject of very interesting researches by Zsigmondy and Siedentopf. Fig. 2 clearly illustrates the principle of the ultramicroscope. The solution to be examined is placed in the glass cell at *b* and is strongly illuminated by a converging beam of light. On observing the lighted-up solution by the microscope at right angles to the path of the beam the colloid substance present in the solution is visible as brightly illuminated particles. The methods of

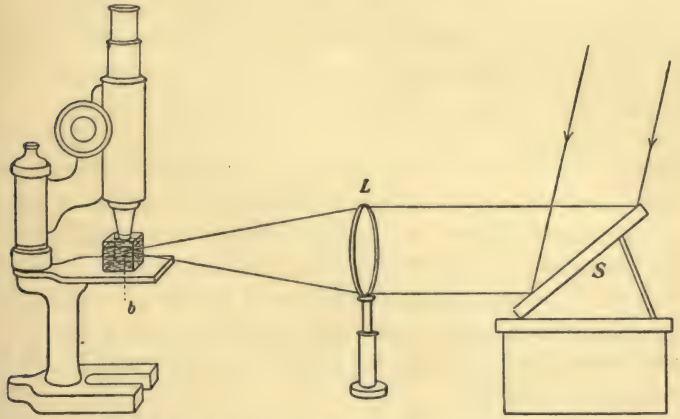


FIG. 2.—THE PRINCIPLE OF THE ULTRAMICROSCOPE.[1]

producing a brilliant converging beam of light, and the construction of the observation cell have in practice been improved and rendered more compact and precise, but Fig. 2 sufficiently illustrates the principle employed.

By means of the ultramicroscope particles are rendered visible which are far smaller than any that can be seen under the ordinary microscope. Thus, for example, if an ordinary blood-corpuscle be represented by a circle three inches in

[1] Reproduced by permission from Zsigmondy's work, *Zur Erkenntniss der Kolloide.*

diameter, a particle of colloidal gold to the same magnification would be barely visible as a minute dot, but yet it can be distinguished by means of the ultramicroscope.

(2) Colloids can readily be precipitated from solution, usually by acidification, by the addition of solutions of various salts or by the introduction of other colloids.

Certain colloids when once so precipitated are only brought into solution again with difficulty, others readily pass into solution if the precipitating agent is removed, e.g., by dialysis, or if its effect is diminished by dilution. These two classes of colloids are distinguished as *irreversible* and *reversible* respectively. The difference in behaviour is probably mainly due to differences in the sizes of the precipitated particles.

A characteristic example of an irreversible colloid is the soluble silica already referred to. On addition of hydrochloric acid or salt solution to the aqueous solution of silica the latter gelatinises and cannot readily be brought into solution again.

A colloid when in solution in water, as in the case of the unprecipitated silica, is frequently referred to as a *hydrosol*; when precipitated in a gelatinous or anhydrous form it is known as a *hydrogel*.

Many enzymes are typical *reversible* colloids. They can be precipitated from their aqueous solutions by means of alcohol, but redissolve in water if the alcohol is removed by filtration.

True colloids conduct electricity very slightly, if at all; in fact, under the influence of the electric current, they move as a whole towards one pole or the other.

The precipitation of one colloid by another has been shown to be connected with the electrical condition of the respective substances. An electro-positive colloid will precipitate an electro-negative colloid, and *vice versâ*.

The precipitation of organic colloids by gelatinous mineral hydroxides which is made use of in the clarification of sewage

is an interesting instance of the mutual precipitation of colloids.

The coagulation of 'toxins' by 'precipitins' in serum therapy is based on the same property.

It is probable that we have here also to do with the attractive action of extended surfaces, such as are presented by gelatinous precipitates, whereby not only colloids but also to a certain extent crystalloids are withdrawn from solution. This attractive effect is known generally as *absorption*.

Colloids exercise a very low osmotic pressure, and consequently are assumed to have a very high molecular weight.

The latter characteristic is of considerable importance in considering the changes taking place in a cell. Modern research has shown that substances, such as ordinary salt, which allow the passage of electricity when they are dissolved in water exist, at any rate in dilute solution, in a state of dissociation, and the dissociated *ions*, as they are termed, obey in dilute solution the laws of gaseous particles. They will tend rapidly to diffuse throughout the solution.

If, therefore, a dilute solution of salt is enclosed in a vessel with semi-permeable walls,[1] i.e., walls which are permeable to the molecules of the solvent but not to those of the dissolved substance, there is a tendency for the ions to extend, they cannot pass through the sides of the vessel; but if the latter be placed in clean water there will be a tendency for the water to enter and thus a pressure will be created in the interior of the vessel; this is known as the *osmotic pressure*. This will obviously depend on the concentration of the salt solution. In dilute solutions it is proportional to the number of the molecules of the dissolved salt present in a given volume of the solution.

In order that chemical activity may go on in the cell, it is evident that it must be possible for crystalloidal bodies to

[1] A porous pot, in the pores of which copper ferrocyanide has been precipitated, forms such a semi-permeable septum.

enter and leave the cell through the cell wall; the rate of interchange of substances will depend on the difference of osmotic pressure within and without the cell.

In fact, a very delicate method of determining differences of osmotic pressure consists in immersing certain plant cells in different solutions and examining the cells microscopically.

If the osmotic pressure of the solution is greater than that of the cell contents, the cell protoplasm will contract and leave the walls of the cell, a phenomenon known as *plasmolysis*.

If the protoplasm within the cells just begins to show signs of contracting, it may be taken that the osmotic pressure is equal on the two sides of the cell wall. Such solutions are said to be *isotonic*, i.e., the number of molecules present in equal volumes of the solutions within and without the cell, or the molecular concentration of the dissolved substances, is such that they exercise the same osmotic pressure. The changes taking place in the cell must consist in the breaking down of colloidal substances, notably albumin, into crystalloidal substances which escape from the cell, and the building up of complex matter from other crystalloidal substances which find entry to the cell. Further, it is obvious that these changes must be analogous to those chemical changes which require the least complexity of chemical conditions, i.e., they must be of the nature of *catalysis*.

It is important, however, to note that while the chemical changes are such as can be produced in many cases in the laboratory, if not by ordinary chemical reagents, at any rate by products or enzymes extracted from the living cell, they only take place in nature when the cell is alive. The precise definition of what is meant by vital action cannot here be attempted; it may, however, be stated that the cell can be looked upon as an energy transformer, in which the energy which is characteristic of living matter, and which may be termed biotic energy, is transformed into chemical activity and eventually into heat in the cell processes.

The simplest kind of living organism is a bacterium or what is popularly known as a microbe; this is a unicellular organism and as a rule specially fitted to bring about certain defined chemical changes. In more complex organisms separate cells are found to have separate functions; thus the cells of the lining of the stomach bring about changes which take place best in an acid medium. In the pancreas, on the other hand, chemical change takes place under alkaline conditions. From many species of cells it is possible to isolate the catalytic substance or enzyme which helps to bring about the change.

While a large number of fermentations are known which can be produced by the action of enzymes, there are others which so far have only been produced by the action of living organisms, such as, e.g., the lactic acid fermentation of sugar or the acetic acid fermentation of alcohol. The alcoholic fermentation of sugar was at one time thought to belong to this class of fermentation. But the experiments of Buchner showed that it was possible to extract a substance from yeast cells which brought about the formation of alcohol and carbonic acid when added to grape sugar; this substance he termed *zymase*, and recent researches by Harden and others have elucidated in a very interesting way the conditions of its activity.

It is probable that other cases where the active enzyme has not yet been discovered will be found on further investigation to resolve themselves in a similar manner.

At the same time it should be pointed out that the activity of the cell is of a complex nature, and it is probable that the living organism is concerned in two distinct modes of activity, i.e., in maintaining its body substance and in developing energy for growth and reproduction. Thus, broadly speaking, in the animal body the processes of digestion are concerned with the maintenance of the body substance, the processes of respiration with the maintenance of energy. In both

cases the chemical action is probably resolvable ultimately into similar factors, though the nature of the products and the energy or heat changes are different in the two cases.

A simple case which illustrates the difference between what may be termed digestive and respiratory fermentation is afforded by the decomposition of urea in the presence of micro-organisms. The simple fermentative change consists in the transformation of the urea into ammonium carbonate by the addition of a molecule of water, as in the following equation :—

$$CO(NH_2)_2 + 2H_2O = (NH_4)_2CO_3$$

At the same time a portion of the nitrogen is found to be taken up by the organism with simultaneous production of CO_2. The second is a much more complex change than the first and its conditions are not so fully understood, but it is probable that here also we have to do with a chemical change in which intermediate loosely compounded complexes are formed, as in the simpler purely chemical reactions mentioned in the earlier part of the chapter.

Finally, it has been found possible, as already stated, not only to break down substances in the manner indicated through the agency of enzymes, but also to effect syntheses of more complex from less complex compounds. Thus, e.g., Croft Hill has been able to produce isomaltose by the action of the enzyme maltase upon dextrose, as follows :—

$$2C_6H_{12}O_6 - H_2O = C_{12}H_{22}O_{11}$$

This discovery is of very far reaching importance and opens up a wide field of possibilities. Already Emil Fischer and his co-workers have announced the synthesis of certain decomposition products of albumin by means of enzyme action.

In the following pages the attempt will be made, by means of typical examples, to render clear the methods of investigation which are used in the study of the chemistry of changes

brought about by enzymes or bacteria. Though it will be necessary to refer to certain organisms, the subject will be approached primarily from a chemical standpoint, fermentation being defined as the *chemical change produced by the agency of protoplasm or of a secretion prepared from it.*

CHAPTER II

OUTLINES OF BACTERIOLOGICAL TECHNIQUE

Bacteriological and enzyme chemistry is essentially the chemistry of the single cell; biological chemistry and physiological chemistry in the wider sense deal with the changes taking place in higher organisms, which consist of collections of cells of varying and interdependent functions. We have therefore only to consider the chemical changes brought about by the simplest organisms, which if not actually unicellular are only very slightly differentiated; or with the chemistry of specific cells of higher organisms. Moreover, from the chemical point of view, the form of the organism, and its method of growth and development, are of less importance than the chemical changes it brings about.

The following pages deal with the methods of recognition and cultivation of the simplest organisms, the subject being treated in quite a general manner. For the detailed methods used in the recognition of specific organisms, text books on bacteriology should be consulted.

The micro-organisms whose chemical activities have to be studied may be divided into three groups, viz. :—

I. Bacteria;
II. Yeasts;
III. Moulds.

Bacteria (Fig. 3, I. and I^{A}).—These are the lowest forms of

vegetable life. Under a high-power microscope they appear as minute round dots, rods or threads ; they multiply either by splitting into two (that is, by *fission* or *cell division*), or by the

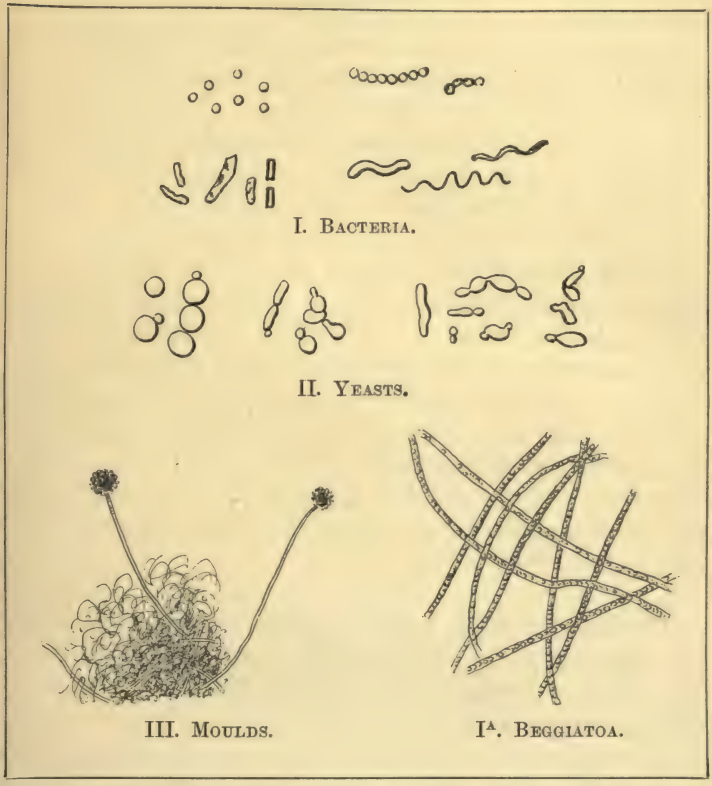


Fig. 3.—Bacteria, Yeasts, Moulds, and Beggiatoa.

production of small protuberances, which separate eventually from the main organism and develop into fresh organisms similar to the parent organism ; this method of reproduction is known as *spore formation*. Bacteria are colourless, that is,

they contain no chlorophyll; they all possess an envelope or capsule consisting probably of cellulose or allied substances. For these various reasons they are classed among the fission fungi, and from their method of reproduction are known as *schizomycetes*.

They are further divided according to their main differences in form into the following subdivisions :—

1. *Coccaceae*, round cells ;
2. *Bacteriaceae*, rods and threads ;
3. *Leptotricheae* } Higher bacteria.
4. *Cladotricheae* }

Yeasts (Fig. 3, II.).—These are closely allied to the bacteria, differing mainly in their method of reproduction. This consists in the formation of small daughter cells or buds which are extruded from the parent cell, a process known as *budding*. Their chemical functions are also more complex, a single yeast cell being able to bring about a number of different chemical changes. As they are mainly capable of growing in a saccharine medium, they are known generally as *saccharomycetes*. The characteristic form of yeast cells with buds is shown in Fig. 3, II.

Moulds (Fig. 3, III.).—These are still more highly organised than the bacteria or yeasts ; they are sporing organisms. The spores or *conidia* give rise to long threads of cylindrical cells forming a network known as *mycelia*. From these cells special seed-bearing organs known as *hyphae* or *thalli* develop. From these organs the moulds derive their general name of *hyphomycetes*.

Thus a mould which at first has a fine thread-like appearance, on further growth will be seen to be covered with minute dots, which are often of a darker colour than the mycelia ; these on microscopical examination will be found to be the hyphae ; in the case, e.g., of *aspergillus niger* the hyphae are

black. According to the form of the hyphae the moulds are divided into four divisions, viz. :—

1. Mucorineae ;
2. Aspergillinae ;
3. Penicilliaceae ;
4. Oidaceae.

All three classes of organisms, bacteria, yeasts, and moulds, occur very widely distributed in nature. They are always most abundant where there is the needful food supply. It is a matter of common knowledge that meat goes bad if long exposed to the air, that jam if uncovered develops mould, that milk becomes sour, that sewage or excretal matter becomes offensive if allowed to accumulate. These changes are due to micro-organisms either originally present in the decomposing substance, or carried in air and deposited on substances capable of putrefactive change, which themselves thus become sources of infection. The presence of bacteria in the air can be demonstrated by exposing a slice of potato for some time in a room. In the course of forty-eight hours or so small spots or centres of growth will appear, which can be recognised as colonies of bacteria or as moulds by methods shortly to be described.

Certain organisms are capable of producing chemical changes in the bodies of higher living organisms, and have been found to accompany the development of specific diseases ; such organisms are termed *pathogenic*.

Other organisms perform exceedingly useful functions. It is scarcely necessary to refer to the technical importance of yeasts in the brewing industries. Special varieties of bacteria are concerned in the production of vinegar and the ripening of cheese, or are useful at certain stages in the manufacture of leather in the tannery. The harmless disposal of refuse matter from men and animals is effected largely by the activity of bacteria, and the processes of agriculture are increasingly found to depend upon the activity of the organisms in the soil ;

they are therefore well described by Percy Frankland as ' our secret friends and foes.'

For the purpose of studying the precise chemical changes effected by a single organism it is necessary to obtain it in *pure culture*, that is, free from admixture of any other organism. The earliest method for accomplishing this, such as was used by Pasteur and Lister, was the method of *dilution*. A small portion of the solution containing the mixture of organisms was transferred to a second portion of the same solution rendered sterile by heat, and after development of the organisms, a small portion of this solution was again transplanted, and so on, until a growth was obtained consisting of only one species of organism, arrived at through a process of natural selection. Such a method is exceedingly tedious, but it is surprising what great advances in knowledge were made by its means. The method of *plate culture* described by Koch in 1881 is much more rapid and certain. Koch introduced a solution containing bacteria into a mixture of suitable nutritive substances thickened with gelatine, the mixture being kept at a temperature slightly above the melting point of gelatine ; on pouring the gelatine culture medium on to a plate and allowing the gelatine to set, wherever a micro-organism was present it developed *in situ*, forming a small cluster or *colony*, which could be picked out and transferred to a similar gelatine culture medium, and if necessary re-plated until only one species of organism was found to be present upon the gelatine plate. The form of plate now generally used is known from its inventor as a Petri dish, and consists, as shown in Fig. 5 *a*, of two shallow glass dishes, fitting into one another, the larger serving as a cover for the smaller, into which the gelatine is poured.

Different culture media have been found to be necessary for different organisms, but all require nitrogen in some form together with certain mineral salts, especially phosphates.

It is of the greatest importance in the preparation of

culture media, and in all operations concerned with the investigations of micro-organisms, to be able to insure *sterility*,

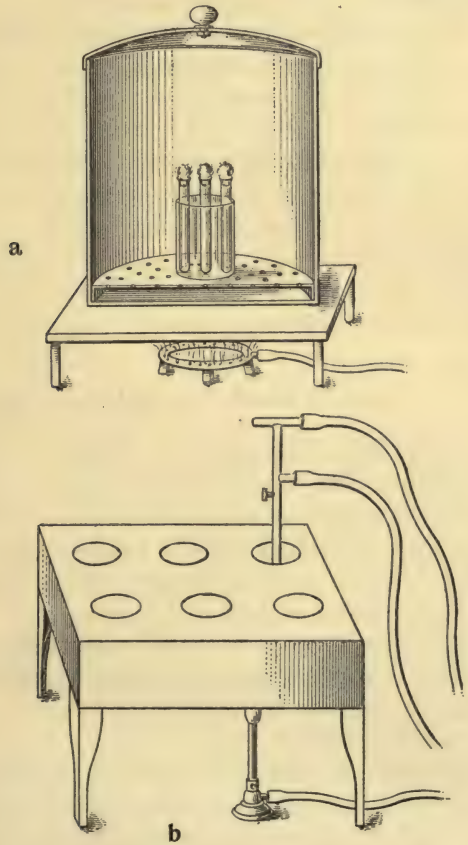


FIG. 4.—(*a*) STERILISER (IN SECTION); (*b*) WATER-BATH.

that is, to insure that no organisms are present in, or gain access to, the medium except those which it is intended to study. A culture medium can generally be effectively

sterilised by exposure to moist steam for about twenty minutes, especially if the operation is repeated in forty-eight hours. In this way any spores which are specially resistant to sterilisation, and which may have escaped the first heating, will have had time to develop, and the adult organism will be killed by the second heating. For sterilising media or apparatus in this manner, a very simple form of steriliser will suffice, which is illustrated in Fig. 4 *a*. It consists of a large semicircular tin can, with ordinary cover, and provided with a perforated false bottom of tin plate about an inch from the bottom of the can. About half-an-inch in depth of water is placed in the bottom of the can, which can be quickly boiled by the flame of a Bunsen burner beneath, the whole can being thus filled with moist steam.

Culture Media for Bacteria

Broth or bouillon.—The basis of most media, suitable for cultivating bacteria, is broth or ' bouillon.' This is made by boiling up one pound of finely minced lean beef free from fat or gristle with one litre of water in a large flask and straining through muslin ; five grammes of salt (sodium chloride) and ten grammes of peptone are added, and the mixture boiled for five minutes. The liquid is rendered very faintly alkaline with carbonate of soda, made up to a litre if necessary with fresh water, the neck of the flask plugged with cotton wool, and the whole sterilised.

Nutrient gelatine is made by dissolving 100 grammes (or 150 grammes if a rather high melting-point is required) of gelatine in 100 c.c. of broth. The gelatine should be first soaked in water to render it easily soluble and the whole volume of gelatine broth made up to 1100 c.c. If necessary, the solution after addition of the gelatine can be clarified by warming on the water bath with the white of one egg. The whole is then filtered through a pleated filter paper in a hot

funnel into a sterile flask, the neck of which is packed with cotton wool. Such a medium is known as G.P.B., gelatine peptone bouillon, 10 to 15 per cent., according to the gelatine added. A medium of this composition will furnish nitrogen and carbon from the albumen and peptone; the necessary salts are also present in the meat extract.

Instead of using actual minced beef 'bouillon,' it is often more convenient to make up a medium directly with Liebig's Extract of Meat. The following formula has been found satisfactory for occasional investigation in a Sewage Works Laboratory. Ingredients:—

Liebig's Meat Extract	9 grammes
Witte's Peptone	9 „
Sodium chloride	4·5 „
Distilled water	900 „
Gelatine	100 „

The meat extract, peptone, salt and water are boiled for a quarter of an hour, and the gelatine gradually added as it dissolves. The whole is allowed to cool (to 50° C. approx.) and neutralised with about 30 c.c. of a 4 per cent. solution of caustic soda (NaOH). The white of an egg is mixed with an equal volume of water and added to the neutralised liquid. The mixture is placed in the steam bath for one hour and 1·5 grammes soda crystals added.

After a further forty minutes in the steam bath the liquid is filtered through a hot water filter as described.[1]

The melted medium is carefully poured, preferably from a separating funnel, into a series of sterile test tubes (cf. Fig. 5 *b*); about 10 c.c. are added to each test tube, care

[1] For very exact work, e.g. differentiation of species, etc., very careful neutralisation of the media is necessary, for the details of which special text books should be consulted. It may be mentioned that the alkalinity or acidity of a medium is often expressed in the number of c.c. of normal acid or soda required for neutralisation, a — sign being used to denote alkalinity and a + sign to denote acidity.

being taken not to allow any medium to run down the sides. The test tubes are plugged with cotton wool, stacked in wire cages and sterilised in the steam bath for twenty minutes on

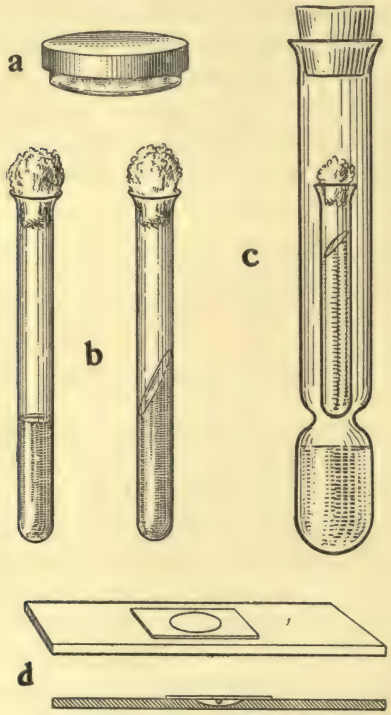


Fig. 5.—Apparatus for Bacteriological Culture.

three successive days. The medium should remain perfectly clear after sterilisation, and the tubes are then ready for use.

Agar medium.—For cultures to be grown at a high temperature, *agar agar*, a Japanese product made from a species of marine alga, is used instead of gelatine in the above process. This medium can be heated to 40° C. without melting.

Starch gelatine.—For the purpose of detection of the enzyme *amylase* among the products of bacteria or other growing cells, 2 per cent. of soluble starch is thoroughly mixed with the melted gelatine medium. The starch in this case should first be boiled with water to a clear paste in order to obtain a homogeneous mixture with the nutrient gelatine.

Silica jelly.—Certain organisms will not grow on ordinary nutrient gelatine, and a method was devised by Kühne and by Percy Frankland in which gelatinous silica is used instead of gelatine, the medium being entirely free from organic matter.

The method of preparation is as follows :—

Two solutions of the following composition are prepared :—

(*a*)	Ammonium sulphate	0·4 gramme
	Magnesium sulphate	0·05 ,,
	Calcium chloride	trace
	Distilled water	50·0 c.c.
(*b*)	Potassium phosphate	0·1 gramme
	Sodium carbonate	0·75 ,,
	Distilled water	50·0 c.c.

These two solutions are rendered sterile by the usual method, after which they are mixed.

A sterile solution of dialysed silicic acid is now prepared as follows : A solution of potassium or sodium silicate is poured into dilute hydrochloric acid ; the mixture is then placed in a dialyser, the outside of which is kept surrounded with running water during the first day, and subsequently with distilled water, which is frequently changed until it yields no trace of turbidity with silver nitrate, thus showing the whole of the chlorides to have been extracted. The contents of the dialyser, if the solution of alkaline silicate originally employed was not too strong, will be quite clear. This liquid is then poured into a flask and concentrated by boiling until it is of such a strength that it is found that, on cooling a little of the solution and mixing it with one-third of its volume of the above mixed

alkaline solution, it readily gelatinises on standing. When the solution of silicic acid is found to give this result, it is cooled, and one-third to one-half of its volume of the mixed alkaline solutions (*a* and *b*) are added, the solutions well mixed and at once poured into Petri dishes or flat-bottomed flasks. The medium should gelatinise in from five to fifteen minutes. The material containing the organisms for examination is introduced and thoroughly mixed, before gelatinisation takes place ; or a streak culture may be made on the surface after the medium has solidified.

As this method has been used for the study of the very important organisms of nitrification, its method of preparation is of special interest.

It will be understood that for the study of special organisms various additions to the typical gelatine or agar media can be made. Thus it is characteristic of certain bacteria, especially, e.g., of *B. coli*, the typical organism of sewage pollution, to produce acid from glucose and other sugars; when therefore glucose and litmus are added to the medium the reddening of the litmus indicates acid formation.

The following medium has been suggested by Dr. MacConkey, and has been largely used for the detection of Bacillus coli in polluted water :—

Sodium taurocholate	.. ..	0·5 gramme.
Glucose	.. .. ..	0·5 „
Peptone	.. .. ..	2·0 „
Water	.. .. ..	..100·0 c.c.

The constituents are heated together, filtered and tinted with litmus solution. The medium is then poured into test tubes and a small inverted fermentation tube placed in each, to serve as a trap for any gas evolved. The tubes are then sterilised in the usual way.

A certain number of bacteria are found only to develop in absence of air; such organisms are classed as *anaerobic* in

contradistinction to those which thrive in presence of oxygen or air. In order to cultivate such bacteria it is necessary to remove the oxygen from above the medium; this can be done most simply by enclosing the culture tube in a larger tube (Fig. 5 *c*) or receptacle containing alkaline pyrogallate of soda, which has the property of rapidly absorbing oxygen. An even simpler method is to fill the tube nearly to the top with medium, and after inoculation to fill up the remaining space with vaseline.

Culture Media for Yeasts.—In the case of yeasts, *wort* gelatine is a more suitable medium than ordinary nutrient gelatine; in this case, instead of bouillon, boiled hot wort, obtainable from a brewery, may be used advantageously; the wort should be diluted with water to a specific gravity of about 1050. The wort must be filtered until it is quite bright, and should remain free from deposit after sterilisation. To prepare wort gelatine 100 grammes of gelatine are added to a litre of the wort and the whole clarified, filtered and sterilised in the same manner as ordinary G.P.B.

Culture Medium for Moulds.—Moulds will grow on nearly all the media so far considered. A solution specially suited for their development is known as Raulin's solution. It is prepared as follows:—

Water	1500	grammes
Cane-sugar	70	,,
Tartaric acid	4	,,
Ammonium phosphate	0·60	,,
Magnesium carbonate	0·40	,,
Ammonium sulphate	0·25	,,
Zinc sulphate	0·07	,,
Ferrous sulphate	0·07	,,
Potassium silicate	0·07	,,

To prepare a Pure Culture of Bacteria.—In transferring

small quantities of material from one medium to another, that is for purposes of inoculation, short lengths of platinum wire mounted in glass rods as in Fig. 6 *a* are used; for small quantities of liquid a wire with a small loop at the end is employed. With a little care loops can be made which will take up almost exactly a milligram, that is 0·001 c.c. of liquid.

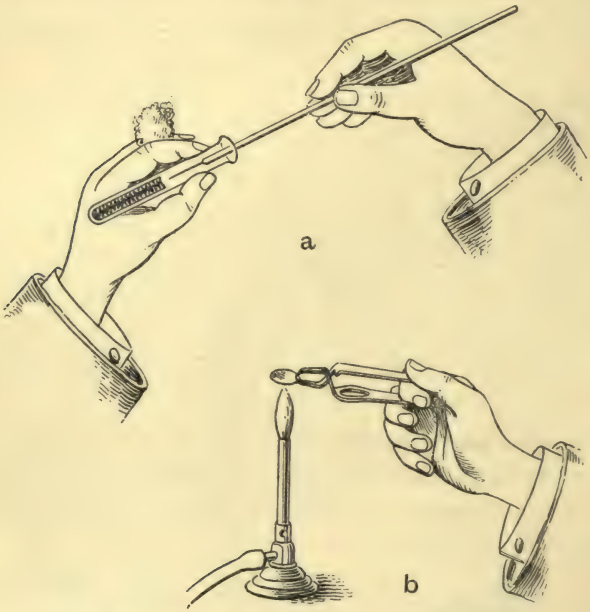


FIG. 6.—(*a*) METHOD OF INOCULATING THE CULTURE MEDIUM; (*b*) FIXING.

For transferring colonies of bacteria a small hook is made at the end of the wire. To inoculate a test tube of gelatine the cotton-wool plug is first sterilised by singeing in the Bunsen flame, is removed by a pair of forceps similarly sterilised, and held between the first and second fingers of the left hand, while the test tube is held between the first finger and thumb (Fig. 6 *a*). The platinum wire, after having

been sterilised by passing through the flame, is dipped into the solution to be examined and then inserted into the gelatine to about half the depth and then withdrawn, the plug of cotton wool again singed and then replaced. Such a culture is known as a stab culture, and is chiefly useful when inoculating from a pure cultivation. If the culture is a mixed one, the gelatine is melted before removing the cotton-wool plug, by allowing the tube to stand for a few minutes in a beaker of water which has been heated to a temperature some ten or twenty degrees above the melting-point of the gelatine. After inoculation and mixing the culture with the melted gelatine, the latter is poured into a sterile Petri dish.

The gelatine is allowed to set in the Petri dish, which is then placed in a moist chamber. The latter is a similar glass vessel of a much larger size, in which some moist blotting-paper or a small Petri dish of water has been placed.

In order to accelerate the growth of organisms on the gelatine in the Petri dish it may be necessary to place the latter in an *incubator*.

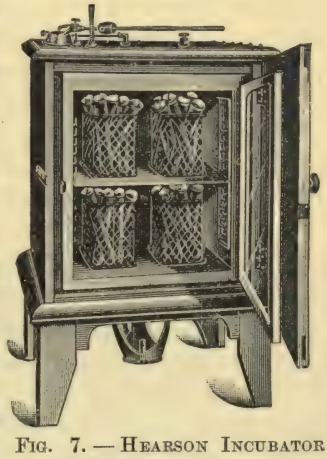


Fig. 7. — Hearson Incubator with Thermo-Regulator. (Messrs. Flatters & Garnett, Ltd.).

The incubator consists essentially of a water-jacketed chamber heated by a gas flame, the size of which, and consequently the temperature produced, can be very exactly regulated by a thermostat. A very satisfactory form of incubator is the Hearson incubator shown in Fig. 7, though less expensive arrangements are obtainable. A set of instructions for adjusting the temperature of the Hearson incubator is issued with the

apparatus. After twenty-four hours the Petri dish should be examined, and signs of the development of colonies will then be probably apparent, though it is generally necessary to allow at least two days to elapse before making the sub-culture. Specific subcultures are best made when the number of colonies on the plate does not exceed 100; it is generally, therefore, best to make two or three plates by transferring a loop full of the inoculated and melted gelatine from the first culture tube to a second and similarly to a third, plates being poured in each case. Well-defined colonies having been obtained on the plate culture, separate colonies can be removed by means of the platinum hook and transferred to a tube of gelatine, there to develop.

For the proper carrying out of these operations, manipulative practice is necessary, in order to avoid accidental infection by extraneous organisms from the air, etc., and also to acquire rapidity and dexterity of handling. It is wise to consider always that everything not actually sterilised is liable to be a source of infection; thus a platinum wire after being laid down on the bench must be re-sterilised, and cotton-wool plugs re-singed after being held between the fingers. Such manipulative details soon become a matter of habit.

Examination of Bacteria under the Microscope.—As already mentioned, a high power is necessary for a satisfactory examination of bacteria. Under a $\frac{1}{12}$ inch oil-immersion lens it is possible to observe them either in the living condition, in a drop culture or as a stained preparation. To examine them in drop culture a small portion of growth either from a plate or tube culture is removed by means of the platinum loop, and quickly mixed with a drop of water on the under side of an ordinary microscopic cover glass, which is then placed on a specially made slide with a depression ground into it (Fig. 5 *d*). On placing the cover slide with the drop on the under side over the depression, the bacteria can be observed.

This method of examination is particularly useful to determine whether the bacteria are capable of movement or not, that is, whether they belong to the class of *motile* bacteria. Bacteria are more simply observed when they are dried and stained with suitable dyes, which render them more clearly observable. There are a number of methods in use for staining bacteria, varying according to the medium in which they are observed, especially, e.g., in tissues, and also for the purpose of bringing out such features as the *flagellae* or thread-like processes, which are characteristic of certain organisms, e.g., the typhoid bacillus. Special methods also are necessary for staining spores. It will be sufficient here briefly to indicate a simple method of staining a pure culture. A carefully cleaned cover glass is taken, and held in a pair of specially constructed forceps, a drop of clean water is placed on the slip and a small portion of the culture mixed with the water and spread in a thin film over the glass by means of a sterile platinum wire ; the film is now carefully dried by passing the glass several times through a Bunsen flame with the film uppermost (Fig. 6 *b*). The cover glass should never be made hotter than can be easily borne by the finger if the under side of the glass is pressed down on it. When the film is dry a drop of stain is placed on the slide, ordinary magenta (rose-aniline) or gentian violet are commonly used. The stain is allowed to remain for a minute or two in contact with the glass and then washed off in a gentle stream of water or by immersion in a large volume of clean water. The preparation is again carefully dried, and a drop of Canada balsam placed on the film side of the cover glass, which is then carefully placed in contact with the ordinary mounting slide. With a little care only such a quantity of Canada balsam is dropped on to the cover glass as will just suffice to reach to its edge when it is pressed down upon the mounting slide. Care should be taken to remove all air bubbles from between the cover glass and the slide.

Preparation of a Pure Culture of Yeast.—It is possible in the case of yeast actually to separate a single cell from the rest of the culture and inoculate suitable media from this single cell. This method, which is of great technical importance in the control of the various fermentations due to yeast, was introduced by Hansen. The following description is based upon that given in Brown's ' Laboratory Studies,' p. 160.

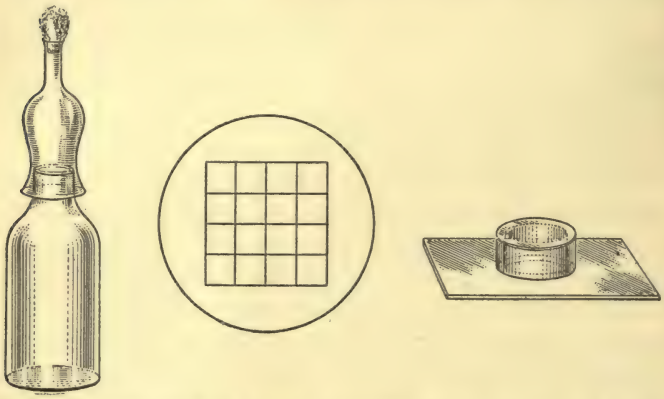


FIG. 8.—FREUDENREICH FLASK, SQUARED COVER GLASS, AND MOIST CHAMBER, FOR YEAST CULTURE.

The following are the requisites for the method :—

A sterilised glass plate and bell jar, or other cover.
Sterilised glass rods.
Sterilised Bötcher chamber.
Sterilised cover glass divided into numbered squares.
Freudenreich flasks of sterilised wort gelatine and of sterilised water.

The Freudenreich flask, the moist chamber and the squared cover glass are shown in Fig. 8. Mix a drop of fresh yeast with sterilised water in a Freudenreich flask, shake well and dilute still further by transferring a drop of the mixture to a

second flask of water. Again mix by shaking, and if the liquid then appears slightly opalescent the right dilution has probably been obtained; transfer a drop of the mixture to a Freudenreich flask containing wort gelatine and mix thoroughly. Then spread a drop of the wort gelatine mixture in a thin layer on the cover glass by means of a glass rod, and place the glass on the glass plate underneath the bell jar and leave until the gelatine is set. Prepare a Bötcher chamber by placing a small drop of water at the bottom of the well and smearing the edge of the ring with vaseline, next reverse the glass with the gelatine film and adjust it to the ring of the chamber; the preparation should then be transferred to the microscope for examination. The lowest-power objective with which the yeast cells can be distinctly seen should be employed. For the purpose of obtaining colonies those cells are chosen which are several millimetres apart from other cells, and their position must be carefully recorded, a diagram being made to indicate the position of the cells chosen.

After marking the position of several cells keep the culture at a temperature of about 20°, and examine it from day to day with the microscope, as the cells multiply, in order to be sure that no cells in the immediate vicinity of the colonies have been overlooked. When the colonies are large enough a pure culture in wort may be obtained from each colony by inoculation in the manner described for gelatine plate culture.

Permanent preparations sufficient to show the general form of the yeast cells can be stained and mounted in a similar manner to bacteria; special methods are necessary to render clearly visible the inner structure of the cell and to stain spores.

Examination of Mould Culture.—Suitable culture media can be inoculated with moulds in a manner similar to the methods used for bacteria. As moulds are aerobic organisms, the method of inoculation on gelatine may be used, in which

case a slight scratch is made on the surface of the gelatine slope (Fig. 5 *b*, p. 24) by means of a platinum hook infected with the organisms, i.e., what is called a 'streak culture.' Growths are of course best obtained when the hyphae are well matured; mould cultivations can be examined in the hanging drop and their stages of growth and developments studied therein.

For the preparation of permanent specimens of moulds some modifications are necessary in the usual staining process. Owing to the presence on their surface of a very thin layer of fat, moulds are not easily moistened with water. Before mounting, therefore, a portion of the mould intended for examination is immersed in alcohol, to which a little ammonia has been added; the mould can then be stained with methylene blue, the filaments of the mycelium and hyphae taking up the colour while the spores remain unstained. Special care must be taken not to overheat the specimens by too rapid drying.

Instead of Canada balsam it is better to use glycerine in the case of organisms such as moulds and algae, infusoria, etc., the cover glass being attached to the slide by a ring of shellac varnish.

CHAPTER III

SOME LEADING CONCEPTIONS IN ORGANIC CHEMISTRY

THE number of chemical substances dealt with in this book is not large, and the chemical reactions involved are not really difficult to follow, even for those who do not possess an extensive acquaintance with organic chemistry, but some understanding of the principles which underly the formulæ employed for expressing the composition and structure of organic compounds, and of certain general reactions which these latter undergo, is essential if the following chapters are to be properly understood.

For the benefit, therefore, of the general reader and of those whose studies have been mainly confined to other branches of knowledge, some space may be usefully devoted to the consideration of certain fundamental conceptions in the science of organic chemistry, and to the description of certain typical substances and their characteristic reactions.

According to the atomic theory of the structure of matter, all material substances are supposed to consist ultimately of *atoms*. A substance which can by some method be divided into two or more kinds of matter differing from one another and from the original substance is evidently a compound of more elementary substances. But a substance which has never yet been subdivided into other kinds of matter having properties different from its own is regarded as an *element*. A few such substances are known, and out of them all others are found to be built up. If, then, we imagine a particle of one of these 'elements,' e.g., of iron, to

be continuously subdivided until upon further subdivision it ceases to exist in the form known to us as iron, at that point we may be said to have reached an 'atom,' one of the ultimate components of matter.

Recent physical researches suggest that the atom itself can be further subdivided into still smaller particles known as electrons, but setting aside this possibility, for the purposes of the chemist it suffices to define the atom as the smallest existing particle of an element.

This idea of the atomic structure of matter is a very old one and was held by the ancients, and entered largely into the conceptions of Robert Boyle and other chemical philosophers.

It is to the genius of Dalton that we owe a development of the atomic theory, which converted it from a more or less barren speculation into a fundamental and fruitful conception. Dalton was able to show that the atom of any given element was characterised by a definite and unalterable *weight* which, while too small to be expressed by absolute numbers, could be referred to in terms of the weight of the lightest then known element, viz., hydrogen, which was taken as unity; thus the atom of iron, e.g., has been found to be 56 times as heavy as the atom of hydrogen.

Dalton used symbols, somewhat akin to the old alchemical symbols, viz., circles, hemispheres and the like, for expressing the ultimate atoms and elements. It was the great Swedish chemist, Berzelius, who introduced the much more convenient method of referring to elements, either by their initial letters, or by the initial letter together with a second significant letter. These are known as the *symbols* of the elements; thus the symbol H signifies one part by weight of hydrogen, the symbol O sixteen parts by weight of oxygen.

In order to obtain true values for these relative weights of the elements, which should really express the weights of their atoms as compared with the weight of an atom of hydrogen, it was necessary to extend the conception of

Dalton and to conceive of chemical substances as being made up of aggregations of atoms which are known as *molecules*.

Now in considering the various states of matter it is evident that it is in the *gaseous* state that the molecules or atoms are most widely separated ; thus, e.g., we know that a comparatively small volume of water will give rise on boiling to a considerable volume of steam. And it is from the study of chemical substances in the gaseous state that our fundamental conceptions of the properties of atoms and molecules and of their relative weights have been chiefly derived.

Before Dalton's time Boyle discovered that various gases, though they might differ in composition, obeyed certain simple laws. Thus Boyle found that if the pressure upon a gas was doubled, its volume at the same temperature was halved, and the statement that the volume of a gas varies inversely with the pressure is known as *Boyle's law*. The same generalisation was made by the Frenchman Mariotte. It was further found by Gay Lussac that all gases expanded equally for equal increments of temperature.

Although later researches have shown that the laws of Boyle and Mariotte and of Gay Lussac only hold strictly within certain limits of temperature and pressure, yet they afford clear evidence that gases possess essentially the same general physical properties whatever be their composition.

When it was further discovered by Gay Lussac that a given volume of oxygen, say, when compared with a given volume of hydrogen under the same conditions of temperature and pressure, was always sixteen times the weight of the hydrogen, the conclusion was inevitable that a definite relation existed between the volume of the gas and the number of atoms in it.

A satisfactory explanation of the properties of gases, and of the relations which exist between the weights of equal volumes of gases differing in composition, was afforded by the Italian chemist, Avogadro, who enunciated the law that *equal*

volumes of all gases under the same conditions of temperature and pressure contain the same number of molecules. Avogadro's conception of molecules served to explain certain discrepancies met with when comparing the weights of equal volumes of different gases: thus, e.g., if the weights of equal volumes of hydrogen and oxygen and of steam be compared—always, of course, under the same conditions of temperature and pressure—it will be found that the ratio of the weights is as follows, viz., H = 1, O = 16, and steam = 9.

It was further found that two volumes of hydrogen combined with one volume of oxygen to form two volumes of steam. Now it is evident that each of the two volumes of steam contains an equal proportion of oxygen, inasmuch as their weights and physical properties are identical. By introducing the conception of molecules, Avogadro enabled a clear conception to be formed of the action taking place. He assumed that the molecule of oxygen contained at least two atoms, one of which combined with hydrogen to form a molecule of steam. We may represent the union of two volumes of hydrogen with one volume of oxygen to form two volumes of steam in the following manner:—

H_2	H_2	+	O_2	=	H_2O	H_2O
2	2		32		18	18

Taking hydrogen as unit, the weights of the molecules will be represented by the figures below the squares, and we thus see how it is that if a volume of hydrogen is taken as weighing 1, the same volume of oxygen will weigh 16, and the same volume of steam 9. Assuming the molecule of hydrogen to contain two atoms, the molecular weight of all other substances will be represented by the weight of their vapour when compared under identical conditions with an equal volume of hydrogen whose weight is taken as two.

We thus reach a very important fundamental conception, viz., that of the weight of a molecule of a substance in terms of the weight of a molecule of hydrogen.

The difference between molecules and atoms receives confirmation from the properties of elements in what is called the *nascent state*, i.e., at the moment of their release from combination.

Thus if gaseous hydrogen is passed, e.g., through a yellow solution of ferric chloride, no change takes place; if, however, the hydrogen is evolved actually in the solution by inserting, e.g., a strip of zinc, the ferric chloride is rapidly reduced with formation of a colourless ferrous salt containing less chlorine than the ferric chloride. The hydrogen in the nascent state combines with the chlorine of the latter according to the following equation :—

$$\underset{\text{Yellow ferric chloride}}{FeCl_3} + H = \underset{\text{Colourless ferrous chloride}}{FeCl_2} + HCl$$

This is a typical instance of a process known generally as *reduction*, when oxygen, or its equivalent, is removed from a compound.

The oxidising properties of such substances as ozone and hydrogen peroxide are due to the liberation of oxygen from them in the nascent state. Ozone is considered to be a condensed form of oxygen containing three atoms in the molecule; on coming in contact with oxidisable matter the third atom of oxygen is liberated and ordinary oxygen with two atoms in the molecule is set free, thus :—

$$O_3 + \text{metal} = O_2 + \text{metallic oxide}$$

Similarly hydrogen peroxide (H_2O_2) readily loses one atom of oxygen with liberation of ordinary water, H_2O.

As a matter of fact ozone and hydrogen peroxide are mutually destructive when they are brought together, for the

loosely combined oxygen atoms in the respective molecules combine together to form a molecule of ordinary oxygen, thus :—

$$O_3 + H_2O_2 = H_2O + 2O_2$$

These are typical cases of *oxidation*, the opposite process to reduction.

We shall see later that this special activity of nascent oxygen is of very great importance in connection with a set of changes brought about by a class of enzymes known as *oxidases*.

The study of the action of elements in the nascent state leads to the conclusion that the atom of an element is in general incapable of a separate existence, and the *atom* has therefore been defined as the smallest portion of an element which can enter into or be expelled from a compound.

A *molecule* is defined as the smallest portion of an element or compound which is capable of a separate existence.

Certain exceptional cases exist where the molecular weight of an element is found to be identical with its atomic weight, but these do not affect the general conclusions.

We may now proceed to the application of these fundamental chemical laws to that branch of the science known as organic chemistry, so called because it deals with the substances elaborated to a large extent by living or organic matter, as distinguished from the constituents of the inorganic or mineral world.

It was at one time thought that organic compounds, properly speaking, could only be produced by vital energy. The synthesis of a characteristic vital product, viz., *urea*, by Wöhler in 1828 broke down this distinction, and since then, out of the countless substances included under the science of organic chemistry, although many are natural products, many have only been prepared in the laboratory and are of purely scientific interest. One characteristic all these substances

possess in common, they all contain carbon, and perhaps the best definition of organic chemistry is, the *chemistry of the carbon compounds*. It is remarkable that the compounds of carbon by far exceed in number the compounds of all the other elements, and the reason for this is to be sought in the nature of the carbon atom itself. In order to understand this we must consider a further general property of atoms, viz., what is known as their *valency*, and for this purpose we must clearly understand the meaning of, and the method of determining, a *molecular formula*.

We have already seen how, by determining the weights of equal volumes of substances in the gaseous state, as compared with the weight of an equal volume of hydrogen, it is possible to determine the weight of a molecule of the substance. By suitable methods of analysis we can determine also the proportion by weight of any element in that compound and thus obtain its molecular formula, just as we have found that the molecular formula for steam is H_2O. Again, by burning a known weight of carbon in oxygen, determining the weight of carbon dioxide produced, and by knowing also the weight of a volume of this gas as compared with the weight of an equal volume of oxygen, we find that 12 parts of carbon unite with 32 parts by weight of oxygen to form a gas the molecular weight of which is 44, and consequently its molecular formula is CO_2. Knowing thus the molecular weight of steam and of carbon dioxide and their molecular formulæ, viz., H_2O and CO_2, we are in a position to determine the molecular formulæ of many organic compounds.

On burning a given weight of a substance containing carbon and hydrogen, the carbon is burnt to CO_2, and the hydrogen to H_2O, which may be respectively weighed; from the weights of CO_2 and H_2O formed, we can calculate the weight of carbon found in the original compound taken, and thus obtain its percentage composition. This method of analysis

is carried out in practice by heating a weighed quantity of the substance to be analysed in a small porcelain boat placed in a tube about a yard long (Fig. 9) filled with granulated oxide of copper, and through which a current of oxygen or air can be passed. The whole tube is heated in a furnace, and any partially burned vapour of the substance which escapes direct combustion is finally oxidised by passing over the red-hot copper oxide. The water is retained in a tube containing calcium chloride, which readily absorbs moisture, and the CO_2 is retained in specially devised bulbs filled with caustic potash, which are weighed before and after the analysis. This process is known as *combustion analysis* and is regularly employed in laboratories devoted to organic chemistry. Special methods, of course, are made use of in the determination of elements other than carbon and hydrogen, e.g., nitrogen, phosphorus, or sulphur. Oxygen is usually determined by difference, i.e., by deducting the weights of all the other elements present from the weight of the substance originally taken, when the remainder, if any, is assumed to be oxygen. The determination of the percentage composition of the substance from combustion analysis will be made clear by the following example :—



Fig. 9.—Apparatus for Combustion Analysis.

0·2 grm. of a substance yielded on analysis 0·290 grm. CO_2 and 0·12 grm. H_2O.

Now in every 44 parts CO_2 there are 12 parts C, therefore in 0·29 grm. CO_2 there will be :—

$$\frac{0{\cdot}29 \times 12}{44} = 0{\cdot}079 \text{ parts C}$$

Similarly in every 18 parts H_2O there are 2 parts H; therefore in 0·12 grm. H_2O there will be :—

$$\frac{0{\cdot}12 \times 2}{18} = 0{\cdot}013 \text{ parts H}$$

Together the C and H make up 0·079 + 0·013 = 0·092 of the total weight, 0·2 grm., of substance taken; the remainder, 0·108, is assumed to be oxygen.

Converting these proportions to percentages we have :—

$$\frac{0{\cdot}079 \times 100}{0{\cdot}2} = 39{\cdot}5 \text{ per cent. carbon.}$$

$$\frac{0{\cdot}013 \times 100}{0{\cdot}2} = 6{\cdot}5 \quad \text{,,} \quad \text{,,} \quad \text{hydrogen.}$$

$$\frac{0{\cdot}108 \times 100}{0{\cdot}2} = 54{\cdot}0 \quad \text{,,} \quad \text{,,} \quad \text{oxygen.}$$

From the percentage composition we can readily calculate the *empirical* formula of the substance, i.e., the simple ratio of the number of atoms of each element to each other, by calculating how many times 12 parts by weight of carbon, 1 part by weight of hydrogen or 16 parts of oxygen, etc., are contained in the percentage amounts, viz. :—

$$\frac{39{\cdot}5}{12} = 3{\cdot}3 \text{ parts of carbon.}$$

$$\frac{6{\cdot}5}{1} = 6{\cdot}5 \text{ parts of hydrogen.}$$

$$\frac{54{\cdot}6}{16} = 3{\cdot}4 \text{ parts of oxygen.}$$

The lowest ratio of these numbers, i.e., the *empirical* formula, is obviously CH_2O, the slight errors in the experimental results being neglected.

In order now to determine the molecular formula of a compound we need to know its molecular weight; this is readily obtained by determining the weight of a known volume of its

vapour as compared with the weight of an equal volume of hydrogen.[1] The number of molecules being the same in the two equal volumes according to Avogadro's law, then, assuming the weight of a molecule of hydrogen to be 2, the *molecular weight* of the substance is twice the vapour density, and the *molecular formula* can therefore now be readily deduced from the *empirical formula*. Thus, supposing that the vapour density of the substance, whose empirical formula was calculated above, was found to be 44, this gives a molecular weight of $44 \times 2 = 88$. The molecular formula will be $C_2H_4O_2$ as the atoms are in the same ratio as in the empirical formula, and the sum of their atomic weights equals 88.

There are of course a great many substances which cannot be vaporised without decomposition; in such cases it is impossible to determine their molecular weights, and consequently their molecular formulæ, by measurement of their vapour density as compared with hydrogen. It has, however, been shown by the experiments of Van't Hoff, Raoult and others, that in dilute solutions the molecules of the dissolved substance behave as if they were in the gaseous state, and a specific effect is produced on the melting and boiling-point of the solvent, proportional to the molecular weight of the dissolved substance. By determining the rise of boiling-point, or the lowering of the melting-point of a solvent, produced by a known weight of the dissolved substance, and comparing these values with those obtained when an equal weight of a substance of known molecular weight is dissolved, the molecular weight of the first substance can be deduced.

Various other means of a somewhat indirect character are made use of in certain special cases; e.g., the determination of the *osmotic pressure* of a solution of known concentration may be employed as indicated in Chapter I.

[1] In practice the weight of a given volume of the vapour is compared, in specially devised apparatus, with the weight of an equal volume of air, and the hydrogen ratio calculated from the result.

This somewhat lengthy description of the methods and arguments involved in arriving at the molecular formula for an organic compound has been entered into, because it appears of fundamental importance that the real meaning of a molecular formula should be properly understood, as all other developments in regard to the molecular structure of compounds depend upon this.

A molecular formula tells us how many atoms of each constituent element are present in the molecule of the compound. It tells us nothing, however, as to the way in which these atoms may be combined within a molecule. The extraordinary advances which modern chemistry has made in the study of the arrangement of the atoms within the molecule, a study which must necessarily precede a systematic attempt to build up these molecules from their constituent elements, naturally had to begin with the study of the simplest compounds. Supposing we take the following simple compounds of carbon, whose molecular weight and molecular formulæ are easily ascertained by the methods already indicated :—

Carbon monoxide, CO ;
Carbon dioxide, CO_2 ;
Methane, CH_4 ;
Chloroform, $CHCl_3$;
Hydrocyanic acid, HCN ;

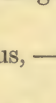
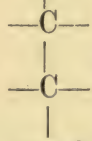
we see that one atom of carbon is able to combine with one or two atoms of oxygen ; with four atoms of hydrogen ; or with one atom of hydrogen and one atom of nitrogen. We also know that one atom of oxygen combines with two atoms of hydrogen to form water, H_2O ; that one atom of hydrogen combines with one atom of chlorine to form hydrochloric acid gas, HCl ; further that one atom of nitrogen combines with three atoms of hydrogen to form ammonia, NH_3.

If we study the formulæ of the five compounds of carbon given in the above list in the light of these facts, we shall see

that the carbon is attached to elements which are equivalent in every case but one to four atoms of hydrogen ; the exception is carbon monoxide, where only one atom of oxygen, equivalent, that is, to two atoms of hydrogen, is attached to the carbon. Carbon monoxide, however, as is commonly known, is a combustible gas, burning with a blue flame to form CO_2, a compound in which again the carbon is attached to two other atoms, together equivalent to four atoms of hydrogen. Moreover carbon monoxide can combine with two atoms of chlorine to form a compound known as carbonyl chloride, $COCl_2$, where again the carbon is combined with atoms which are together equivalent to four atoms of hydrogen. Such examples might be multiplied, with the result that it can be shown that one atom of carbon is always capable of combining with four atoms of hydrogen or their equivalent. Incidentally we have learnt also that one atom of chlorine is capable of taking the place of one atom of hydrogen ; one atom of oxygen is capable of taking the place of two atoms of hydrogen ; one atom of nitrogen is capable of taking the place of three atoms of hydrogen. This atom-replacing power of the elements is known as their *valency*. We speak of chlorine and hydrogen as being monovalent, of oxygen as divalent, of nitrogen as trivalent, and of carbon as tetravalent. Where the atom of an element does not exercise its full valency, an *unsaturated* compound results, such, e.g., as carbon monoxide.

Throughout the vast range of organic chemistry the carbon atom is always tetravalent ; where it apparently is not tetravalent, further atoms can always be taken into combination till saturation results. Victor Meyer indeed was accustomed to define organic chemistry as 'the chemistry of constant valency,' because such constancy is not so apparent among the elements which build up the mineral kingdom.

We must now consider the second very important property of the carbon atom. Not only will the carbon atom, as we have seen, combine with hydrogen, with chlorine, etc., it will

also combine with *itself*. This fact lies at the foundation of Kekulé's *law of the linking of atoms*, which is one of the main foundation stones of modern organic chemistry. The genesis of this idea of Kekulé's was singular. He tells us that it came to him more or less as a dream. As he was sitting half asleep by the fire, he seemed to see the atoms executing a mazy dance, till suddenly some of them separated themselves into chains, while others joined themselves in rings. He sat up all night working out the consequences of this dream. Very briefly it came to this, that if we consider a single carbon atom with its tetrad valency, exercising a power of combination with four atoms of hydrogen or their equivalent, it may be symbolically written thus,  ; if another atom joins itself to this, a compound will be formed with a skeleton structure of this kind, viz.,  and so on. Each of the vacant 'bonds,' as they may be termed, can be combined with hydrogen or other elements, and we can easily see that as we go on adding carbon atoms, for each carbon atom two hydrogen atoms or their equivalent can be also added. Thus we get what is known as an *homologous series*. Supposing the bonds in the above case to be combined with hydrogen, we obtain the series, C_nH_{2n+2}; this is the series of *paraffin* hydrocarbons, the initial members of which are :—

Methane, CH_4, followed by
Ethane, C_2H_6,
Propane, C_3H_8,
Butane, C_4H_{10}, etc.

If two adjacent carbon bonds in such a chain be left

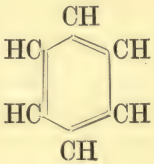
unsaturated, we then get the series of the *olefine* hydrocarbons of the general formula C_nH_{2n}, e.g. :—

Ethylene, C_2H_4,
Propylene, C_3H_6,
Butylene, C_4H_8, etc.

The initial member of this series should of course be methylene CH_2, but all efforts to prepare it result in the formation of ethylene or dimethylene.

A further elimination of hydrogen results in the series C_nH_{2n-2}, the initial member of which is acetylene, C_2H_2.

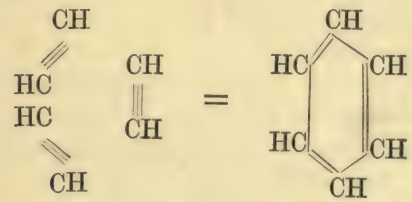
The next great series resulting from Kekulé's generalisation are the *ring* hydrocarbons, of which the best known member is *benzene*. Kekulé represented benzene by the following formula :—



The proof of the ring formation in benzene is a very beautiful instance of the method of determining what is known as the *constitutional* formula of an organic compound. Inasmuch as the structure of benzene as indicated by Kekulé's formula is a symmetrical one, it should follow that whichever of the hydrogen atoms is replaced by chlorine the same monochlorbenzene should result. As a matter of fact, however, monochlorbenzene is prepared; only one monochlorbenzene has ever been obtained. It has indeed been possible by a series of reactions, too complex to be here considered, systematically to replace one atom of hydrogen after another in benzene, and, as has been stated, whichever atom is replaced only one monochlorbenzene results.

A formula such as Kekulé's formula for benzene, which

gives a symbolic representation of the relation of the atoms in the molecule one to another, is known as a *constitutional formula.* That these formulæ do, as a matter of fact, bear some relation to an actual reality in nature, is shown by the circumstance that, once a constitutional formula has been correctly established, the artificial production of the substance is generally only a matter of time. Thus, to take the case of benzene itself, its formula suggests that if three molecules of acetylene C_2H_2 could be induced to combine, benzene C_6H_6 would result. On passing acetylene through a red-hot tube benzene is actually produced, the reaction being represented as follows :—



It goes without saying that before any conclusion can be drawn as to the composition or constitution of a compound, it is essential that it should be obtained pure. The methods in use in organic chemistry for obtaining compounds in the pure state resolve themselves into *crystallisation* and *distillation.*

Crystallisation is effected by evaporating a solution of the substance in suitable solvents either at the ordinary atmospheric pressure or in vacuo. The crystals first deposited are usually the purest; by redissolving these and repeating the process pure crystals are eventually obtained. This process is known as *fractional crystallisation.* Crystallisation is often brought about by combining the substance to be purified with some other body with which it will form a crystallisable compound. A notable instance of this method is the case of many of the sugars, which by themselves form

difficultly crystallisable syrups; they can be combined with a substance known as phenyl hydrazine to form well-defined crystalline compounds.

The crystalline compound is pure when it has a constant melting-point; that is, if the melting-point of the substance is determined and it is redissolved and recrystallised, and the melting-point of the crystals again determined, the two melting-point determinations should be the same.

To purify a substance by *distillation*, it can be distilled either at the ordinary atmospheric pressure or under reduced pressure, so long as the temperature of the vapour remains constant; if a rise of the thermometer is observed during distillation, it means that some substance other than the lower boiling substance is being distilled over. By repeating the distillation of the portions distilled over between various limits of temperature, a distillate is finally obtained having a constant boiling-point; such a process is known as *fractional distillation*. The separation of the products of petroleum by distillation on the large scale is a good instance of this process. It is characteristic of a pure compound that it has a *constant boiling-point*.

It may not be superfluous here to emphasise the fact that to the chemist a substance can only be considered to be a definite chemical entity when it satisfies one of three conditions :—

1. It has a definite crystalline form,

or

2. It has a constant melting-point,

or

3. It has a constant boiling-point.

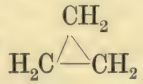
Many of the substances met with in the chemistry of vital processes, more especially the derivatives of albumin, do not satisfy these conditions. Such substances can be differentiated one from another by their general chemical and physical properties, and by the products of their decom-

position under defined conditions, but they cannot be looked upon as chemical individuals in the same sense as compounds which fulfil one of the above-mentioned requirements.

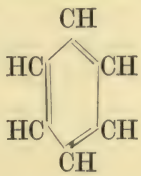
The determination of the constitutional formulæ of the countless substances met with in the study of organic chemistry shows that they can be classified under three heads.

1. *Aliphatic compounds*, viz., all open chain compounds both saturated and unsaturated, viz., the paraffin, olefine, acetylene, etc., hydrocarbons already referred to, and their derivatives.

2. *Isocyclic compounds*.—All compounds containing closed chains formed by the union of carbon atoms only, viz., derivatives of polymethylene hydrocarbons, consisting of rings formed by three or more CH_2 groups; thus :—

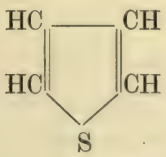
trimethylene 

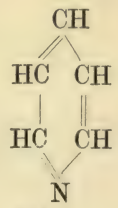
or substances derived from benzene



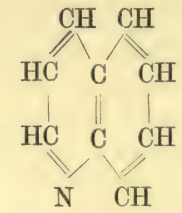
and from hydrocarbons containing more than one ring such as naphthalene, anthracene, etc.

3. *Heterocyclic compounds*.—All compounds containing closed chains, having other atoms in addition to carbon atoms, viz. :—

thiophene 

pyridine 

quinoline



etc., and their derivatives.

From all of these root compounds derivatives can be built up by well-defined processes, and these derivatives are characterised by containing certain groups of atoms which are easily recognisable by their reactions.

It will be useful at this stage to consider the more important classes of derivatives and their reactions in a highly general manner. A knowledge of organic chemistry really consists in being familiar with certain general reactions typical of certain specific atomic groups, rather than in a detailed acquaintance with individual compounds. In what follows, therefore, reference will be made mainly to those atomic groupings, the knowledge of whose properties will be useful in the study of the substances to be considered in the later chapters of the book.

Alcohols.—These are derivatives of aliphatic hydrocarbons characterised by the presence of the group —OH, known as the *hydroxyl* group. The simplest alcohol is *methyl* alcohol, CH_3OH, a hydroxyl derivative of methane, CH_4. Ordinary alcohol is the next member of the series, viz., hydroxy-ethane or ethyl alcohol, CH_3CH_2OH (or C_2H_5OH). Alcohols may be divided into three classes :—

Primary alcohols of the general formula $R—CH_2OH$;
Secondary alcohols of the general formula $R_2{=}CHOH$;
Tertiary alcohols of the general formula $R_3{\equiv}C—OH$.

Alcohols are capable of combining with mineral acids to form salts, thus :—

$$RCH_2OH + HCl = RCl + H_2O$$

Aldehydes and Ketones.—The first product of the oxidation of an alcohol is either an *aldehyde* or a *ketone*. Primary alcohols yield aldehydes, thus :—

$$\underset{\text{Primary alcohol}}{R{-}CH_2OH} + O = \underset{\text{Aldehyde}}{R{-}C\begin{matrix}H\\ \diagup\\ \diagdown\\ O\end{matrix}} + H_2O$$

Secondary alcohols yield ketones :—

$$R_2{=}CHOH + O = R_2{=}C{=}O + H_2O$$

Tertiary alcohols yield mixtures of aldehydes and ketones.

It will be noted that both aldehydes and ketones contain the group $>C{=}O$ which is known as *carbonyl*; in fact aldehydes differ only from ketones in that a complex residue R replaces hydrogen in the latter. The group $>C{=}O$ is a highly reactive group; the German word *reactionsfähig*, or capable of reaction, is perhaps more expressive.

As this group occurs in most of the carbohydrates, certainly in most of the sugars, and possibly in cellulose, it is important that its general reactions should be understood. The more commonly used are the following.

With ammonia an amino compound is produced thus :—

$$R{=}C{=}O + NH_3 = R{=}C\begin{matrix}OH\\ \diagup\\ \diagdown\\ NH_2\end{matrix}$$

With hydrocyanic acid we have the following :—

$$R{=}C{=}O + HCN = R{=}C\begin{matrix}OH\\ \diagup\\ \diagdown\\ CN\end{matrix}$$

With phenyl hydrazine a compound of the following formula $C_6H_5\overset{H}{\diagdown}N{-}NH_2$ we have :—

$$R{=}C{=}O + H_2N{-}NHC_6H_5 = RCN{-}NHC_6H_5 + H_2O$$

Acids.—Upon oxidation the CO group gives rise to an acid, the exact composition of which depends on the elements or groups attached to the carbon. Thus an aldehyde oxidises as follows :—

$$\begin{matrix} R\diagup H \\ \diagdown CO \end{matrix} + O = \begin{matrix} R\diagup OH \\ \diagdown CO \end{matrix}$$

A ketone gives a mixture of acids according to a rather more complex reaction.

The group CO_2H, which is a shortened form of the group $-C\begin{matrix}\diagup OH \\ \diagdown O\end{matrix}$ as written above, is known as the *carboxyl* group, and is characteristic of all organic acids which may be written according to the general formula RCOOH; thus in acetic acid R is represented by the group CH_3 or methyl, and the formula of the acid is CH_3COOH. The substance used as an illustration of the determination of a molecular formula on p. 44 was acetic acid. On reduction with nascent hydrogen the group CO_2H is reconverted to $-C\begin{matrix}\diagup H \\ =O\end{matrix}$ and $-CH_2OH$, i.e., acids give on reduction aldehyde and alcohols.

Esters.—Alcohols combine with organic acids to form what are known as *esters* or ethereal salts; thus ethyl alcohol combines with acetic acid according to the following equation :—

$$C_2H_5OH + CH_3COOH = CH_3COOC_2H_5 + H_2O$$

which may be generalised as follows :—

$$ROH + RCOOH = RCOOR + H_2O$$

It should be noted that these reactions in which salts are formed from alcohols with elimination of water are typical examples of what are known as *reversible* reactions; that is, when a certain amount of water and salt is formed, an equilibrium is attained, and the reverse action tends to take place, resulting in the formation of acid and alcohol. Such reactions are generally written thus :—

$$ROH + RCOOH \rightleftarrows RCOOR + H_2O$$

If it is desired that the reaction should become complete it is necessary to add some substance such as strong sulphuric acid or chloride of zinc which will take up water as it is formed.

It is probable that under specific conditions nearly all chemical reactions are reversible. The case of the esters is interesting as a simple one, which has been carefully studied.

Ethers.—Esters should not be confused with ethers, which are bodies of the general formula $\begin{matrix} R \\ R \end{matrix}\!\!>\!O$, R in this case representing a hydrocarbon residue; thus, in ordinary ether R = the group C_2H_5 or ethyl, and its formula is $\begin{matrix} C_2H_5 \\ C_2H_5 \end{matrix}\!\!>\!O$.

Phenols.—When the group OH is connected directly with a benzene ring, substances known generally as phenolic compounds are produced, the simplest of which is ordinary carbolic acid or phenol,  . On oxidation these substances yield somewhat complicated mixtures and are thus distinguished from ordinary alcohols.

Groups containing Nitrogen.—The simplest compound of nitrogen is of course ordinary ammonia, which has the composition NH_3. Each of these three atoms of hydrogen is capable of being replaced by complex groups of various kinds; moreover, just as ammonia, NH_3, combines with acids, e.g., HCl, to form ammonium chloride, NH_3HCl (or NH_4Cl as it is generally written), so organic derivatives of ammonia also are capable of acting as bases in this way. Ordinary sulphate of quinine is a case in point. Ammonia derivatives are possessed of different properties according, on the one hand, to the number of hydrogen atoms replaced or, on the other, to the character of there placing groups. E.g., if one of the hydrogen atoms is replaced by a hydrocarbon residue we have what are known as amino derivatives, thus :—

CH_3NH_2 is *methyl-amine.*

$C_6H_5NH_2$ is phenylamine or amino-benzene, commonly known as *aniline.*

$\begin{matrix} CH_2NH_2 \\ | \\ COOH \end{matrix}$ is *amino-acetic-acid,* glycocol or glycin, a very important member of the series of amino acids.

If the replacement is effected by an acid residue an acid *amide* results; thus CH_3CONH_2 is known as acetamide.

The well-known substance *urea* is an amide of carbonic acid, $CO\begin{matrix} OH \\ OH \end{matrix}$, and has the formula $CO\begin{matrix} NH_2 \\ NH_2 \end{matrix}$.

The group NH_2, which is thus seen to be formed by the replacement of one hydrogen in ammonia by a complex group, is known as the *amino* group, and like other well-defined groups it can be recognised in a compound by its specific reactions; one of the most important of these is its reaction with nitrous acid, which results in the elimination of nitrogen and

the replacement of the NH_2 group by the hydroxyl group —O—H, thus :—

$$R—HN_2 + HONO = R—OH + N_2 + H_2O$$

When two atoms of hydrogen in ammonia are replaced the group NH is left, which is known as the *imino* group. This also is characterised by its reaction with nitrous acid when substances known as *oximes* are obtained, thus :—

$$R_2NH + HONO = R_2N—NO + H_2O$$

Finally all three hydrogen atoms in ammonia may be replaced and we obtain a *tertiary* amine, R_3N.

Compounds are known which are derived from a combination of two amino groups joined thus :—

$$H_2N—NH_2$$

This substance has been prepared and is known as hydrazine ; its phenyl derivative $C_6H_5HN—NH_2$ has already been mentioned more than once, and is a substance of great importance because of its property of combining with the carbonyl group which occurs in numerous sugars, and of thus giving rise to crystallisable compounds.

Cyanides.—The group CN is an important one because of the facility with which on treatment with water (in presence of acid or alkali) it gives rise to the group COOH, that is, to acids, thus :—

$$RCN + 2H_2O = RCOOH + NH_3$$

Such a process in which one or more molecules of water take part is generally known as *hydrolysis*.

Moreover, on treatment with nascent hydrogen, it is reduced, forming an amino derivative, thus :—

$$RC{\equiv}N + 2H_2 = RC\begin{matrix}{=}H_2\\ -NH_2\end{matrix}$$

In the foregoing paragraphs are given some of the more important atomic groupings which are met with in the substances which form the subject matter of bacteriological and enzyme chemistry ; the point must be emphasised that wherever they occur, and however complicated the atomic groupings may be with which they may be associated, they can always be recognised by their specific reactions.

A short table summarising the reactions of the few typical groupings which have been considered may therefore be found useful (Table I). The unsaturated linkings, it must be understood, may combine with groups of atoms of greater or less complexity symbolised by R.

Constitutional Formulæ.—In determining the constitution of a compound the main problem consists in ascertaining by the reactions given what atomic groupings are present. To take a simple case, a substance is found by the methods already indicated to have the molecular formula CH_4O. Upon oxidation it is found to yield an aldehyde and finally an acid. We conclude, therefore, that it contains the group CH_2OH, and bearing in mind that the carbon atom is uniformly tetravalent, we assign the constitution CH_3OH and write the equation expressing its oxidation as follows :—

$$HCH_2OH + O = HCHO + H_2O$$
$$HCHO + O = HCOOH$$

The substance is, of course, methyl alcohol yielding on oxidation formaldehyde and formic acid.

Isomerism.—A little reflection will already have suggested that it is possible, even though the number of atoms in a molecule may be identical, that the arrangement of atoms within the molecule may differ in different cases. In considering the constitutional formula for benzene, the assumption of a ring arrangement of the carbon atoms in the molecule

Table I

Group	Typical Reaction
$-CH_2OH$ Primary alcohol group	Oxidises to aldehyde $-C\begin{matrix}H\\ \diagup\\ \diagdown\\ O\end{matrix}$ and acid $-CO_2H$
$=CHOH$ Secondary alcohol group	Oxidises to ketone $=CO$ and mixture of acids.
$\geqslant C-OH$ Tertiary alcohol group	Oxidises to mixture of acids.
$=C=O$ Carbonyl group	With ammonia forms $=C\begin{matrix}OH\\ \diagup\\ \diagdown\\ NH_2\end{matrix}$ With hydrocyanic acid forms $=C\begin{matrix}OH\\ \diagup\\ \diagdown\\ CN\end{matrix}$ With phenyl hydrazine forms $=C=N-NHC_6H_5$
$-C\begin{matrix}OH\\ \diagup\\ \diagdown\\ O\end{matrix}$ Carboxyl group	Yields on reduction $-C\begin{matrix}H\\ \diagup\\ \diagdown\\ O\end{matrix}$ and $-C\begin{matrix}H_2\\ \diagup\\ \diagdown\\ OH\end{matrix}$ Combines with alcohols to form *esters* of the general formula $-COOR$
$-NH_2$ Amino group	Replaces H in hydrocarbons to form a primary amine, e.g., CH_3NH_2, methylamine; $C_6H_5-NH_2$, phenylamine or aniline. Replaces hydrogen in an acid to form *amino* acids thus, $\begin{matrix}CH_2NH_2\\ \vert\\ CO_2H\end{matrix}$, amino-acetic. Replaces OH in the carboxyl group of an acid to form an *amide* thus, CH_3CONH_2, acetamide. Treated with nitrous acid yields nitrogen and an alcohol, thus:— $RNH_2 + HONO = ROH + N_2 + H_2O$ The hydrogen in the NH_2 can be further replaced, yielding:— R_2NH Secondary amine; R_3N Tertiary amine
$-C\equiv N$ Cyanogen group	On hydrolysis yields the carboxyl group $-CO_2H$ On reduction yields an amine, RCH_2NH_2

was seen to be justified by the fact that only one monochlorbenzene could be obtained. Many other consequences follow from the ring formation, but the above is one of the simplest, and suffices to distinguish benzene from another possible arrangement of six carbon atoms and six hydrogen atoms to form the molecule C_6H_6 which might be conceived as follows, the atoms of carbon forming a chain :—

$$CH{\equiv}C{-}CH_2{-}CH_2{-}C{\equiv}CH$$

Such a substance does as a matter of fact exist, and is known as dipropinyl; it differs, however, from benzene in that it forms several monochlor derivatives according as chlorine is attached to the first, second or third carbon atom from the end of the chain. Moreover it will be seen that dipropinyl is an acetylene hydrocarbon that readily combines with bromine, the bromine being added to the compound, which then becomes saturated. The first action of bromine upon benzene is one of substitution.

Two compounds such as benzene and dipropinyl, which have the same number of atoms in the molecule but whose atoms are differently arranged, are known as *isomeric* substances, and the phenomenon is spoken of generally as *isomerism*. In order to determine the arrangement of the atoms in the molecule and thus to distinguish between isomeric substances, a systematic study must be made of the typical reactions of such substances.

A simple case may be taken to illustrate the determination of the *constitutional* formula in the case of two substances having the molecular formula C_2H_3N. When these substances are treated with potash one of them yields potassium acetate and ammonia, while the other yields methylamine and potassium formate. These reactions point clearly to the conclusion that in the one case the two carbon atoms must be closely connected, as they reappear together in the molecule of acetic acid; in the other case, one of them is separated from

the other and joined to nitrogen, and so reappears as methylamine. Bearing in mind the underlying assumption that the hydrogen atoms are always monovalent, the carbon atoms always tetravalent, and the nitrogen either trivalent or pentavalent, the above reactions find satisfactory explanation in the following formulæ and equations :—

$$\underset{\text{Cyanide.}}{H_3C{-}C{\equiv}N} + KOH + H_2O = H_3C{-}C(=O){-}OK + NH_3$$

$$\underset{\text{Iso-cyanide.}}{H_3C{-}N{\equiv}C} + KOH + H_2O = H_3C{-}N{=}H_2 + HC(=O){-}O{=}K$$

The first compound is termed a cyanide, the other an *iso*-cyanide.

Another simple but important instance of isomerism may be referred to in illustration, viz., the case of the *lactic acids*. Ordinary lactic acid is produced by the fermentation of milk sugar or lactose; it has the molecular formula $C_3H_6O_3$. Another acid of the same molecular formula exists whose chemical properties are quite different from those possessed by the fermentation lactic acid. The difference between these two acids finds an explanation in the reactions by which they have been artificially prepared, and in the products to which they give rise on oxidation, etc. An acid having chemical properties identical with the fermentation acid is obtained from acetaldehyde by the following typical reactions. By the action of hydrocyanic acid on aldehyde a cyanhydrin is formed :—

$$\begin{array}{l} CH_3 \\ | \\ C{=}O \end{array}\!\!\diagup\!\!H + HCN = \begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ CN \end{array}$$

Upon hydrolysis, according to the general reaction several times referred to, a salt of lactic acid is formed thus :—

$$\begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ CN \end{array} + KOH = \begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ COOK \end{array} + NH_3 + H_2O$$

This acid contains, it will be seen, the group $\begin{array}{l} | \\ CHOH \\ | \end{array}$; upon oxidation, therefore, this will yield the group $=C=O$, and a ketonic acid is formed, thus :—

$$\begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ COOH \end{array} + O = \begin{array}{l} CH_3 \\ | \\ C=O \\ | \\ COOH \end{array} + H_2O$$

It should here be noted that it is customary for the convenient nomenclature of open chain compounds to refer to the C atoms in order as α, β, γ, etc., according as they are one, two, three, etc., removes from the end of the chain. The above lactic acid is therefore known as α lactic acid. It is obvious that the hydroxyl OH group might be attached to the second carbon atom, when a β acid would be obtained. We thus have:

$$(\alpha)\ \begin{array}{l} CH_3 \\ | \\ CHOH \\ | \\ CO_2H \end{array} \qquad (\beta)\ \begin{array}{l} CH_2OH \\ | \\ CH_2 \\ | \\ CO_2H \end{array}$$

As a matter of fact the latter is the second lactic acid above referred to. It is obtained from β iodopropionic acid, which is known to have the constitutional formula

$$\begin{array}{l} CH_2I \\ | \\ CH_2 \\ | \\ CO_2H \end{array}$$

the iodine being replaced by the group OH, through the action of moist silver oxide, AgOH.

The constitutional formula of β-lactic acid is further confirmed by the fact that on oxidation, as would be expected, the group CH_2OH yields finally a carboxyl group CO_2H, and a dibasic acid known as malonic acid is formed, thus :—

$$\begin{array}{l} CH_2OH \\ | \\ CH_2 \\ | \\ CO_2H \end{array} + O_2 = \begin{array}{l} CO_2H \\ | \\ CH_2 \\ | \\ CO_2H \end{array} + H_2O$$

In the next chapter reference will be made to certain other isomeric varieties of lactic acid which cannot be distinguished by any difference in the products of their reactions or in the methods of their preparation ; the present chapter may, however, fitly end at this point with a few words of summary and emphasis.

It will have been sufficiently evident that in such a limited space only a few simple examples have been made use of to illustrate the general principles of the science of organic chemistry. It is of the greatest importance for the proper understanding of any subject involving the use of organic compounds, and the expression of the construction of the compounds by formulæ, that the real meaning of these formulæ should be once for all properly understood. For this reason rather disproportionate space has been taken in the endeavour to make clear the meaning and the methods of determining successively molecular weights and molecular formulæ. To

discuss constitutional formulæ at any length would involve writing a text-book on organic chemistry, but emphasis has been laid on the importance of a knowledge of the reactions of certain fairly simple groups of atoms which occur again and again in the numberless substances which form the subject matter of this science. Finally, one or two very simple instances of the determination of constitutional formulæ have been given. It is thus hoped that even those readers whose knowledge of organic chemistry is limited, may yet be able easily to follow the subsequent chapters of this book.

CHAPTER IV

SPACE-ISOMERISM AND THE CHEMISTRY OF THE SUGARS

Towards the end of the preceding chapter reference was made to certain varieties of lactic acid which could not be distinguished by their chemical reactions and yet whose physical properties were not identical. It is found, e.g., that if ordinary lactic acid produced by fermentation is combined with strychnine, which has the properties of a base and thus forms salts with acids, and if the strychnine compound is allowed to crystallise slowly from solution, the first portions of salt which crystallise out will differ in physical properties from those which are obtained later; the most important difference is in regard to the action of their solutions upon polarised light.

This property of affecting polarised light is one of very great importance, and its study has led to great extensions in our conceptions of molecular structure. Moreover, the effect of certain solutions upon polarised light affords a means of determining the quantity of dissolved substances present in solution. For all these reasons it is important that the fermentation chemist should possess some elementary knowledge at any rate of the subject of the polarisation of light, and of the practical use of the polarimeter, and at this point, therefore, it will be well to make a digression and devote some space to the theory and use of the polarimeter.

The Theory of the Polarimeter

Light is one of the primary forms of energy which reaches this earth from the sun. It is well known that the atmosphere of air surrounding the earth becomes more and more attenuated as the distance from the earth increases, and, in fact, does not extend even in a rarefied form to a greater distance than approximately 200 miles from the surface of the earth; some medium other than the atmosphere must, therefore, be conceived of as a means of transmitting light and other forms of energy from the sun to the earth. This medium, which is thought of as filling all space, has been termed the *luminiferous ether*, for the reason just mentioned. Light is conceived of physically as a wave motion set up in this all-pervading ether. The essential features of *wave motion* can be readily studied by carefully observing the ripples formed when a pebble is thrown into a still pool of water; it will be seen that the water does not move as a whole from the point where the pebble plunged, but that a series of up and down motions takes place in successive portions of water. This can be easily verified by throwing in a few light match stalks, which will be seen merely to move up and down and not to approach the edge to any appreciable extent. The same essential feature of wave motion is clearly seen when the wind blows over a field of wheat; obviously, here individual ears of wheat cannot move beyond certain limits, and there is only a to and fro, or up and down movement. The regular movement within certain limits, such as the water particles or the ears of wheat exhibit under the above circumstances, is known as a *vibration*; the extent of the displacement of any given vibrating particle from its position of rest is known as the *amplitude* of the vibration, and a motion such as we have been considering is known generally as *wave motion.*

A wave length is measured from crest to crest or from hollow to hollow of the wave. If it were possible to set two

waves in motion in the same direction, the crests of one corresponding to the hollows of the other, the vibrations of the wave particles would obviously neutralise one another and all motion would be stopped. Such a phenomenon is termed the *interference* of waves. This interference may be complete, as in the case just mentioned, which would occur when one wave was exactly half a wave length behind the

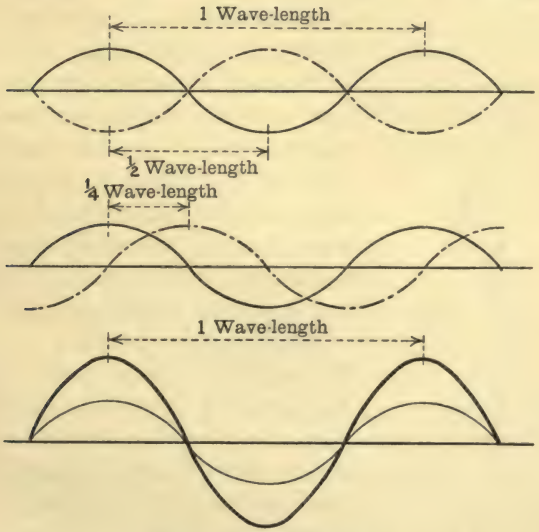


FIG. 10.—WAVE MOTION.

other; it would be less complete if the one wave were a quarter or three-quarters of a wave length behind the other, whereas a difference of a whole wave length would mean that crest reinforced crest and hollow reinforced hollow, and the amplitude of the vibrating particles would be doubled. This is clearly seen from Fig. 10.

It has been found that the physical properties of light receive their full explanation if it is assumed that light

consists in a wave motion of the luminiferous ether; the intensity of light depends on the amplitude of the vibrations, the colour of the light depends on the wave length, which is generally referred to as λ.

Now in an ordinary ray of light the waves are conceived of as following each other in very rapid succession, with constantly varying planes of vibration; thus, e.g., if we imagine a wave motion (Fig. 11) vibrating in one instant of time parallel to AB, the following wave may vibrate along A′B′ and the next along A″B″ and so on. Such a ray of light, therefore, has no two-sidedness, that is, the plane of vibration of its waves cannot be determined; on the other hand, a ray of light all of whose waves pass through AB would be referable definitely to this plane, and such a ray is said to be *polarised.*

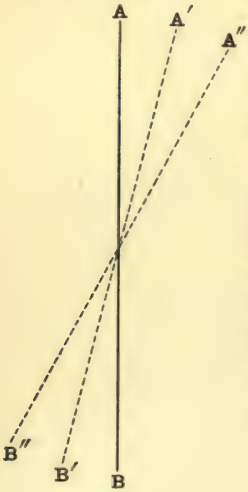


Fig. 11.

The unassisted eye is unable to distinguish between polarised light and ordinary light; it is conceivable that if we could construct a barred screen of sufficient fineness to prevent the passage of all waves except those undulating in a plane parallel to the bars, we should know that the light passing through the screen was polarised in that plane. Now the structure of certain crystals is such that they act somewhat in the manner of such a screen, and compel the waves of light passing through them to vibrate in defined planes. Such a crystal is *tourmaline*; if two pieces of tourmaline cut parallel to the long axis of the crystal are placed at right angles one to the other, opacity results.

For an explanation of this property it will be necessary

more closely to consider what happens when a ray of light passes through the crystalline medium, and for this purpose we may study a crystal of *calcspar*. Calcspar crystallises in beautiful rhombs which are colourless and transparent; unlike a rhomb of glass, however, we shall find that if a crystal of calcspar is placed over an inkspot on a piece of white paper two inkspots will be seen: this is known as the phenomenon of *double refraction*. Calcspar, like tourmaline, belongs to a class of crystals whose density, or the packing of whose particles, is different in different directions. Now it is a simple consequence of the undulatory theory of light, that the velocity of propagation of a wave varies according to the density of the medium, and further that, owing to this alteration of velocity as the wave passes from one medium to another of differing density, alteration of the direction of the wave takes place. As the density of the calcspar crystal is different in different directions the rays vibrating along one axis will emerge from the crystal in a direction differing somewhat from those vibrating in the plane of the other axis; thus we have either two images in the case of the inkspot, or, if we direct a ray of light upon the face of the crystal, two beams will emerge. A further phenomenon is observed; if we slowly rotate the crystal over the inkspot one spot will be found to maintain its position, the other moves round with the crystal, and similarly with the two rays of light. The ray whose position remains unaltered as we move the crystal is known as the *ordinary* ray, because it obeys the ordinary laws of refraction, that is, a constant relation always obtains between the angle of incidence and the angle of refraction. This is not the case with the ray giving rise to the movable image. This ray is therefore referred to as the *extraordinary* ray.

The peculiarity of tourmaline is, that while it breaks up the ray into two, in the same way as the rhomb of calcspar does, the planes of vibration being likewise at right angles

to each other, it has the property of diverting the ordinary ray and only allowing the extraordinary ray to pass, whose vibrations are parallel to the long axis of the crystal. Such a ray is polarised, and a ray which is polarised at right angles to this direction will not pass through the crystal. Consequently when two plates of tourmaline identical in structure are held at right angles no light passes, and it follows also that a plate of tourmaline is capable, as already indicated, of enabling us to recognise whether a ray of light is polarised or not. By means of such a plate of tourmaline it can be shown that the rays issuing from the rhomb of calcspar are polarised at right angles one to the other. The use of tourmaline for studying polarised light is unsatisfactory, owing to the green colour of the crystal and the consequent loss of intensity in the light passing. Obviously a better source of polarised light would be one of the emergent rays from calcspar. If means could be found to cut off one of these rays, the other would remain as a ray of undiminished intensity whose direction of vibration was known. Such a means is found in the Nicol prism. This prism is made by taking an elongated rhomb of calcspar and dividing it so that the plane of division *aa'* forms an angle of 68° with the vertical sides of the rhomb as in Fig. 12, and the two portions are then reunited by a film of Canada balsam. If now a ray of light *cd* impinges upon the shorter face of the prism, double refraction will take place, but the ordinary ray suffers total reflection at the surface of the Canada balsam and so passes out of the crystal in the direction *hl*. The extraordinary ray *def*, on the other hand, suffers no refraction on the surface of the Canada balsam, and so passes on with its direction unaltered,

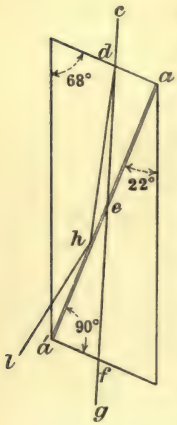


Fig. 12.
Nicol Prism.

and we have thus an emergent polarised ray *fg*. In practice the absorption of the ordinary ray is effected by mounting the Nicol prism in a black mounting. Such a prism is known as a *polariser*. A second similar prism placed parallel to the first will of course allow the ray similarly to pass through ; if held at right angles, on the other hand, it will act in a similar way

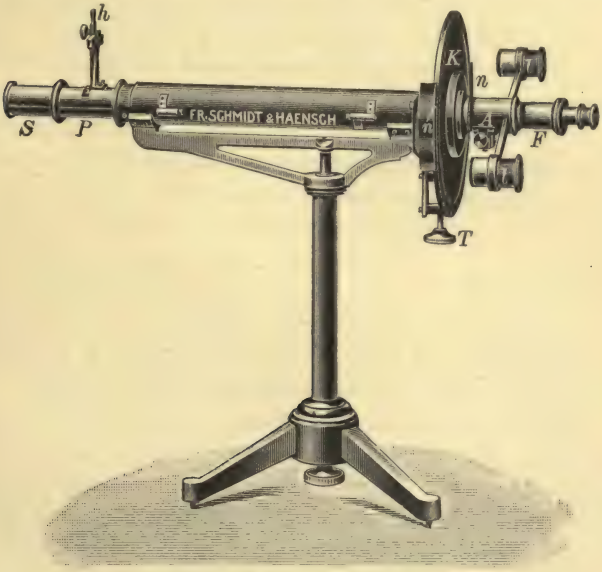


FIG. 13.—POLARIMETER (*Schmidt and Haensch pattern*).

to the cross tourmaline plate, i.e., as a hypothetical barred screen, and will extinguish the ray. The second Nicol prism, as it enables us to recognise the polarised ray emerging from the polariser, is known as the *analyser*. An instrument fitted with these two prisms, together with suitable lenses for observing the ray and with a tube between the prisms in which substances can be placed to observe their effect on the polarised ray, is known as a polarimeter (Fig. 13). Certain details of

construction and methods of use are necessary in such an instrument if accurate results are to be obtained. It is only, it must be remembered, with rays of a given wave length that absolute darkness will, as a rule, be obtained when the Nicols are crossed, because the angle of refraction is different for rays of differing wave length, so that a prism that was cut in such a way that the ordinary violet ray was just totally reflected might not completely cut off the ordinary ray of red light, and so a small proportion would come through even when the Nicols were crossed. It is better, therefore, always to use light of a definite wave length, and for this purpose the yellow light obtained when a compound of sodium, such as a bit of melted carbonate of soda, is held in the flame of a Bunsen burner, is employed; even then the point of complete darkness is not altogether easy to distinguish. It must be remembered that unless the Nicols are exactly at right angles a certain *component* of the vibration will pass through, increasing in amount in proportion as the Nicols become more nearly parallel. This may be rendered clearer by the following diagram; keeping to the analogy of the barred screen, if we assume the barred screen (Fig. 14) placed at an angle to the polarised ray vibrating along AB this vibration will be resolved into two, one, *ba*, parallel to the bars which will pass through and the other, *bc*, at right angles which will be extinguished. Obviously the component passing through will depend on the angle of the barred screen to the polarised ray, and in the diagram *ab* represents the portion of light passing through.

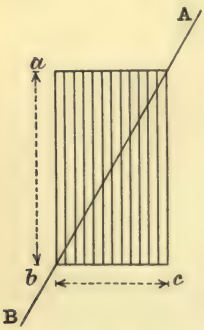


FIG. 14.

In order sharply to define the point of darkness in the polarimeter, half the field of view of the instrument is taken

up with a semicircular plate of quartz, cut in such a direction in reference to the optic axis of the crystal, and of such a thickness that it retards the light passing through by half a wave length. We thus obtain two beams of polarised light, differing in phase by half a wave length. At a certain angle, therefore, interference will take place, as explained earlier, in the case of the rays passing through the quartz plate, and one side of the field will appear completely black and the other completely bright. A position can, however, be found when both sides are completely bright or, on the other hand, completely dark. By a differential arrangement of this sort it is much easier to distinguish the alteration of illumination which occurs on moving the analyser, and we can thus make exact observations of the effect upon the polarised ray of substances placed between the two prisms.

The effect of the quartz plate just referred to is not only to retard the wave by half a length, it also alters its plane of vibration, and, therefore, if such a plate is inserted between crossed Nicols, a certain component of the light passing through the quartz will also pass through the analyser. In order to produce interference and consequent darkness, it is necessary to rotate the analyser through a certain angle in order that the rays passing through the quartz should be brought into the same plane as those passing through the analyser. Other substances besides quartz are capable of altering or rotating the plane of polarisation, even when their *solutions* are placed in the polarimeter, between the two prisms.

For observing the effect of such solutions, a glass tube closed by thick glass discs and screw caps is made use of, tubes of different lengths being used according to the concentration of the liquid to be examined.

In Fig. 15[1] are given the essentials of construction of the Laurent polarimeter.

[1] Adapted by permission from Dr. A. Findlay's *Practical Physical Chemistry.*

L represents a Bunsen flame in which is inserted a bead of carbonate of soda to obtain monochromatic light. A is a lens to render the rays of light parallel, B is the polariser, C and C′ the quartz plate. O is the tube containing the solution to be observed, D the analyser, and EF the telescope.

The Lippich model of polarimeter differs only from the Laurent in having a small Nicol prism to produce the half shadow instead of the quartz plate. The outward appearance of the two instruments is identical, and is shown in Fig. 13. In both cases a light filter consisting either of a solution of potassium bichromate or a crystal of this salt is placed in front of the lens A.

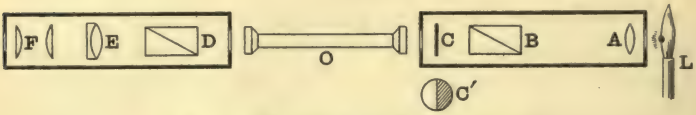


Fig. 15.—Diagram of Polarimeter.

After passing through the polariser and the quartz plate, the light emerges as two beams of polarised light, differing in phase by half a wave length. If the polariser is rotated so that the plane of polarisation forms an angle (θ) with the quartz plate, the planes of polarisation of the two beams will also be inclined at an angle, equal to 2θ. This is the half-shadow angle. On rotating the analyser, a position will be found at which the one beam will be completely, the other only partially, extinguished. The one half of the field of view, therefore, will appear dark, while the other half will still remain light.

The details of practical use of the polarimeter will be better considered in a later chapter; it should here be stated that the angle of rotation of the analyser can be accurately measured

on a circular scale. Further, those substances whose solutions give a right-hand twist to the plane of polarisation looked at from the eye of the observer are known as *dextro-rotatory*, those which twist it in the opposite direction are known as *lævo-rotatory*. Inasmuch as this phenomenon implies that the wave is twisted as it passes through such substances, it is known as *circular polarisation*.

The Relations between Optical Activity and Molecular Structure

The effect of a solution of tartaric acid and sundry other organic substances upon the plane of polarisation was observed

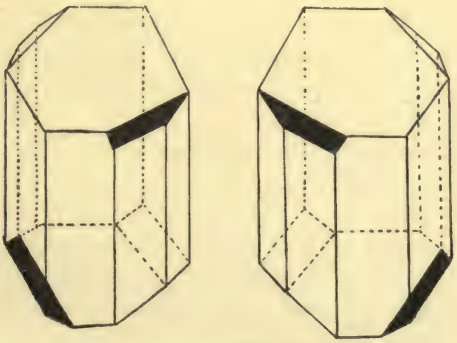


FIG. 16.—EXANTIOMORPHOUS CRYSTALS.

first by Biot in 1838, and he also showed that racemic acid, which has the same composition as tartaric acid, does not possess this optical property. This was confirmed by Mitscherlich in 1844, but it was Pasteur in 1848 who made the first great step in unravelling the cause of this difference. He found that by careful crystallisation of sodium ammonium racemate, a salt which in itself has no action upon polarised light, it was

possible to pick out crystals which differed from each other in structure only as the image in the mirror differs from its real object, or as the right hand differs from the left; thus certain small faces could be seen on one set of crystals on the right hand, whereas the corresponding set of faces on the other crystal were on the left (Fig. 16).[1] One of these crystalline forms turned the plane of polarisation to the right, the other to the left, and the crystals were derivatives respectively of dextro and lævo tartaric acid. When these two forms were crystallised together to form racemate, optical inactivity resulted.

The optical difference in these two modifications of tartaric acid was here clearly referred to a difference in crystalline form. Pasteur at the same time suggested that the cause of the difference lay deeper, viz., in the actual molecular structure of the two acids, that is upon the arrangement of the atoms in their respective molecules. This illuminating suggestion of Pasteur found its full development in the theory of Van't Hoff and Le Bel.

These investigators found that every optically active substance contained within its molecule a carbon atom to which were attached four dissimilar groups; such a carbon atom they referred to as an *asymmetric carbon atom*. In order to explain why such a grouping should give rise to actual physical asymmetry they suggested that the arrangement of the groups must be considered as occurring in *three dimensions*. Now all investigation goes to show that the four combining units, bonds, directions of affinity, or whatever term may be used to express what is symbolised by the four lines attached to the C in the formula of an organic compound, are strictly equivalent. The only way to express this fact in three dimensions is to consider the carbon element as being at the centre C of a regular tetrahedron (Fig. 17), with its

[1] Reproduced from Dr. C. A. Keane's *Modern Organic Chemistry*, by permission of the publishers.

four affinities (shown by the full lines) directed towards the four solid angles (shown dotted), thus :—

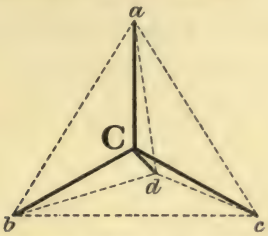


Fig. 17.—The Tetrahedron of the Carbon Atom, in Perspective.

If now the four different groups be attached at each of the four angles of the tetrahedron, say, *a*, *b*, *c* and *d*, it will be seen that a right-hand and left-hand arrangement can be produced thus (Fig. 18) :—

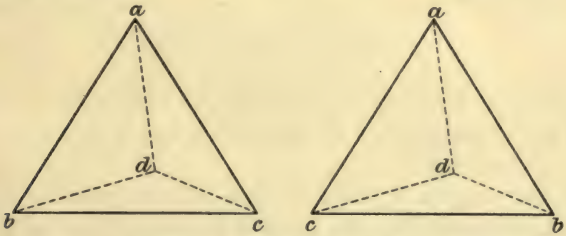


Fig. 18.—Right- and Left-hand Arrangement. [1]

[1] The following useful suggestion is taken from F. J. Moore's *Outlines of Organic Chemistry* (p. 150). The student can readily construct tetrahedral models from pasteboard in the following manner. An equilateral triangle is drawn, each of the three sides bisected and the middle points joined up as shown in Fig. 19. The large triangle is cut out and the corners folded along the dotted lines of the smaller one; the points at the top are joined up by fine wire or gum paper. By marking the corners differently the simpler relations of space isomerism can easily be studied.

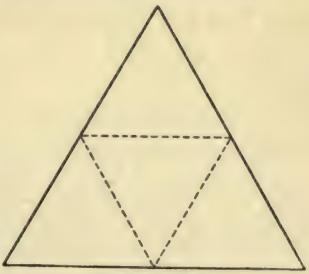


Fig. 19.

These two arrangements cannot be symmetrically superposed; they are what is known as *enantiomorphous*. Such a difference can only occur when all four replacing groups are different, that is to say, when there is an asymmetric carbon atom in the molecule, as in the example just given, *a*, *b*, *c* and *d*, representing the replacing groups.

If the substitution takes place by groups *a*, *a*, *b*, *c*, thus (Fig. 20)—

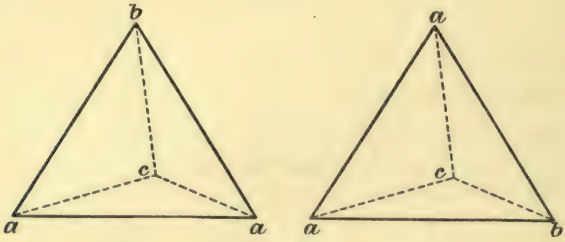


Fig. 20.

it is easy to see that by turning the tetrahedron the two forms are superposable, so that there is no essential difference between them.

We are now in a position more fully to understand how there exists more than one form of lactic acid and of tartaric acid, even though the constitutional formulæ as determined by chemical reactions may be the same; thus in the last chapter it was shown that fermentation lactic acid or *a*-lactic acid had the formula $CH_3\,\mathbf{C}\,HOHCO_2H$, the centre carbon atom is attached to four different groups and is therefore asymmetrical.

It is possible, therefore, to obtain a *dextro*, a *lævo*, and an *inactive* lactic acid. As already stated, these different forms can be obtained by fractional crystallisation of the strychnine salts, and also from the zinc salts. In writing what are known as stereo-chemical formulæ, i.e., formulæ expressive of the space arrangement of the atoms in the molecule, it is convenient,

instead of drawing the actual tetrahedral perspective, to write a projection of the formula on the plane of the paper (Fig. 21), the asymmetric carbon atom being distinguished by a circle round it or by heavy type. Thus the dextro and lævo lactic acid can be written as follows :—

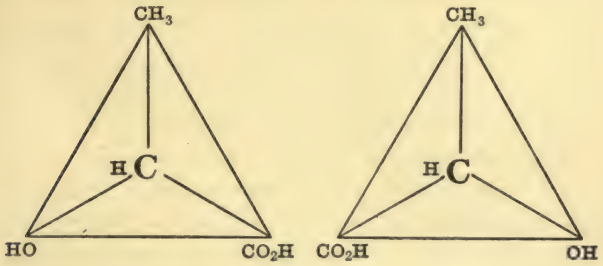
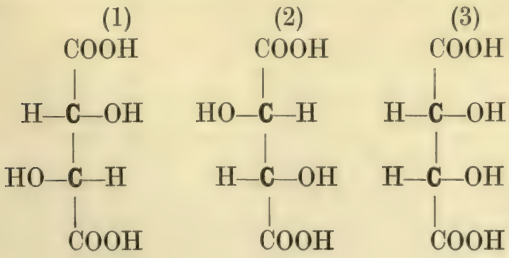


FIG. 21.

or in projection looked at from above :—



The case of the tartaric acids is somewhat more complex, as there are here two asymmetric carbon atoms in the molecules ; the following configurations are therefore possible :—



If the upper and lower halves of each molecule be considered, it can be seen that in formulæ (1) and (2) the upper and the lower halves are not mirror images of each other; both upper and lower halves therefore represent the same optical isomer. Which is actually the formula for dextro or for lævo tartaric acid is a matter of indifference, but both will be optically active and their mixture will form *racemic acid.* On the other hand, in formula (3) the upper and lower halves of the molecule are related as object and mirror image and represent therefore optically opposite groups. We have here intra-molecular compensation and such an acid is optically inactive; it is known as *meso-tartaric* acid. These relations of the four acids can be summarised as follows, *d* and *l* being the opposite optical activities of the two portions of the molecule :—

d	*l*	*d* *l*	*d*
|	|	| |	|
d	*l*	*d* *l*	*l*
Dextro-rotatory	Lævo-rotatory	Racemic	Meso-tartaric

The cases of the lactic and tartaric acids will serve to illustrate the character of the isomerism which is to be found in more complex substances and especially among the sugars, a field of organic chemistry which has been worked out in great detail, mainly by Emil Fischer.

Before passing on to a brief sketch of the chemistry of the sugars it is important that the reader should understand that although the conception of space-isomerism owes its origin to observations connected with the optical activity of substances, yet once the spatial arrangement of the atoms is conceded, and the carbon atom considered always as being the centre of a tetrahedral space, a number of conclusions follow, quite unconnected with the subject of optical activity. Remarkable relations have been found to obtain between the structure of compounds and their stability, which become

clear when actual models are built up in which the tetrahedral arrangement of the carbon affinities is retained.

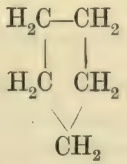
The explosive nature of acetylene derivatives appears to bear some relation to the space formula for carbon, as can be seen by the following space formula for acetylene :—



There is evidently a condition of strain between the two carbon atoms, the line of attraction not being direct between carbon and carbon as in the case, e.g., of a saturated compound, the space formula for ethane being—



Moreover, when a series of carbon atoms is thus joined into a ring, it is found that a differing amount of strain is put upon the bonds, considered for the moment as semi-rigid links, according as the ring contains a different number of carbon atoms. Thus the pentamethylene ring, which may be shortly written thus—



is found to be the most stable arrangement, while rings of more than seven atoms are difficult if not impossible to prepare.

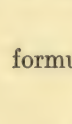
Further it has been found that differences in the constitution of certain *nitrogen* compounds find their best explanation on the assumption of a varying arrangement in three-dimensional space of the groups attached to the *nitrogen*.

Recent researches by Pope and by Kipping have extended the idea of space-isomerism to the derivatives of silicon, tin and other elements. Pope has also recently developed a theory according to which the crystalline form of every substance is minutely dependent upon its molecular structure, thus confirming Pasteur's original suggestion, while approaching the subject from the side of the molecule rather than from the side of the crystal.

These references to recent developments in space-isomerism, or *stereo*-isomerism, have been made because remarkable relationships have been found to exist between the actions of *enzymes* and the stereo-chemical configuration of the molecules of the substances upon which they act; in fact, a very common method for obtaining an active substance from the inactive mixture, which results from ordinary methods of preparation, consists in submitting such an inactive mixture to the action of certain organisms or the enzymes secreted by them, when one modification is generally attacked at a different rate from the other. Further references to this subject, and also to the theory of the natural production of optically active substances, will be made later. We have now to consider a class of substances which perhaps more than any other serve as the basis for extremely important fermentation processes, viz., the sugars.

INTRODUCTION TO THE CHEMISTRY OF THE SUGARS

The term 'sugar' as popularly understood generally refers to cane sugar or preparations made from it. Chemically speaking, however, the word has a much wider application,

and comprises a large number of substances which are classified as *carbohydrates*. A carbohydrate is a compound of carbon with hydrogen and oxygen, the last two elements being in the proportion to form water; the simplest carbohydrate, therefore, would be CH_2O. As a matter of fact this is formaldehyde with the constitutional formula  .

There is considerable evidence for believing that the great family of carbohydrates as found in nature may originate in the first instance from formaldehyde. A suggestion of this sort appears reasonable even when we simply look at the empirical formulæ of the three great classes of carbohydrates generally termed the *mono-saccharoses*, the *di-saccharoses*, and the *poly-saccharoses*. These terms are not altogether satisfactory, because the so-called *mono-saccharoses* include a large number of substances of differing molecular weight and molecular formulæ, all of which have the general formula $C_nH_{2n}O_n$; the best known members of this group are, however, the *hexoses* of the general formula $C_6H_{12}O_6$, and the *di-saccharoses* are so named because by addition of a molecule of water they give rise to two molecules of a hexose. The general formula, therefore, of the di-saccharoses is $C_{12}H_{22}O_{11}$. The *poly-saccharoses* are much more complicated substances whose molecular formulæ are unknown, but they have the general formula $(C_6H_{10}O_5)_n$.

Taking now, for the sake of comparison, an even number of carbon atoms in all three cases we get the following relationship :—

mono-saccharoses (two or more molecules)	$C_{12}H_{24}O_{12}$
di-saccharoses	$C_{12}H_{22}O_{11}$
poly-saccharoses ($n = 2$)	$C_{12}H_{20}O_{10}$

We can thus see at a glance how these important naturally

occurring groups are generally related. The mono-saccharoses can evidently be considered as built up by the combination of a number of molecules of formaldehyde. By elimination of water from two or more molecules of mono-saccharoses, a di-saccharose results, and by further elimination of water poly-saccharoses are obtained. Among the more important members of these various groups may be mentioned, among the saccharoses, *grape sugar* or *glucose*, and *fruit sugar* or *lævulose*; among the di-saccharoses, *cane sugar* (or beet sugar, which has the same composition) and *milk sugar*; among the poly-saccharoses, *starch* and *cellulose*.

Our knowledge of the molecular structure of carbohydrates is naturally greatest in regard to the simplest group, viz., the mono-saccharoses, and inasmuch as sugars belonging to this group are produced by the addition of the elements of water to both di-saccharoses and poly-saccharoses, it is evident that a knowledge of the simpler substances must be of great help towards the ultimate unravelling of the much more complicated chemistry of such substances as starch and cellulose. We may, therefore, proceed to consider the general properties of the carbohydrates of this group.

Mono-saccharoses.—The members of this group of carbohydrates may be described as the first oxidation products of alcohols containing more than one carbon atom each of which has an OH group attached.

The simplest alcohol is of course *methyl alcohol*, HCH_2OH, its first oxidation product is *formaldehyde*, $\begin{matrix} H \\ | \\ C \\ \| \\ O \end{matrix}\!\!\diagup H$, and, as already stated, formaldehyde is the simplest carbohydrate, and may be looked upon as the basal substance of the sugars, although it does not itself exactly fall within the above definition of a mono-saccharose.

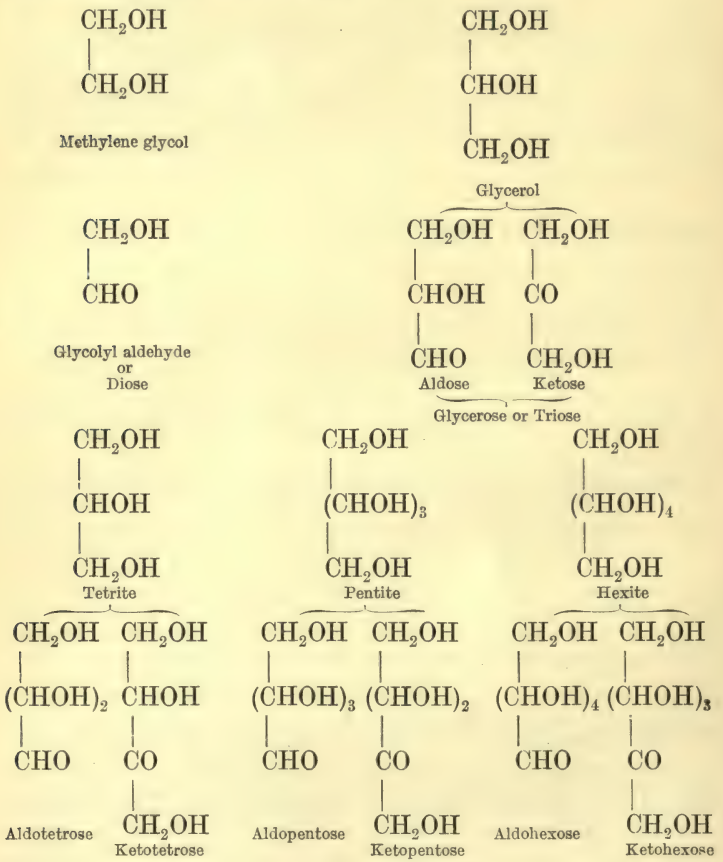
The first *alcohol* which fulfils the definition given above is *glycol*, $\begin{matrix}CH_2OH\\|\\CH_2OH\end{matrix}$, so named from its sweet taste, and the corresponding sugar is glycol-aldehyde, $\begin{matrix}CH_2OH\\|\\CHO\end{matrix}$.

The next member of the series of alcohols is *glycerol* or ordinary glycerine, whose sweet taste is a matter of common knowledge. The formula for glycerol is $\begin{matrix}CH_2OH\\|\\CHOH\\|\\CH_2OH\end{matrix}$, from which it is evident that two first products of oxidation can be obtained, that is, the CH_2OH group may oxidise to an aldehyde group $-C\begin{matrix}H\\\diagup\\=O\end{matrix}$ or the CHOH to $>CO$, the characteristic ketonic group, and thus we have the following relationship :—

CH_2OH	CH_2OH	CH_2OH
|	|	|
CHOH	CHOH	CO
|	|	|
CH_2OH	CHO	CH_2OH
Glycerol	Glycerolaldehyde	Di-oxy-acetone

These last two substances are the first representatives of two important groups of the mono-saccharoses, viz., the *aldoses* and the *ketoses*, the former containing the aldehyde group $-C\begin{matrix}H\\\diagup\\=O\end{matrix}$ and the latter the ketonic group $>C=O$. The sugars it will be noted end in *ose*, and according to the number of carbon atoms they are referred to as biose, triose,

etc., while the corresponding alcohols are generally given the termination *ite* or *ol*; we have, therefore, the following series of alcohols and corresponding sugars, the aldose and ketose form being given in each case :—

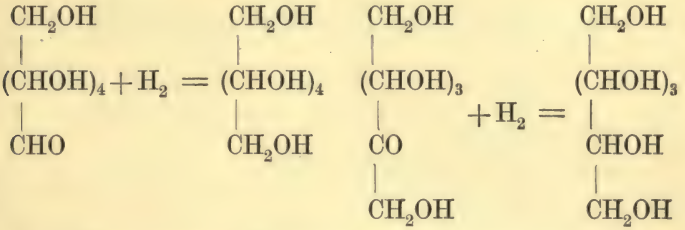


Sugars containing seven, eight or nine atoms of carbon have been obtained, viz., heptoses, octoses and nonoses, but the above list includes those sugars which are met with in

nature and which have a practical as well as a scientific importance, and it is unnecessary, therefore, to extend it further.

All these aldoses and ketoses have certain general reactions by which they can be readily identified :—

1. They are readily reduced by *nascent hydrogen* to the corresponding alcohols, the aldehyde and ketone group being attacked, thus :—



2. *Dilute nitric acid* oxidises aldoses to oxycarboxylic acids, thus :—

$$CH_2OH(CHOH)_4CHO + O = CH_2OH(CHOH)_4CO_2H$$

With ketoses the chain is broken on oxidation :—

$$CH_2OH(CHOH)_3COCH_2OH + 3O = \underset{\text{Tartaric acid}}{CO_2H(CHOH)_2CO_2H} + \underset{\text{Glycollic acid}}{CO_2HCH_2OH}$$

3. *Phenyl-hydrazine* converts both aldoses and ketoses into hydrazones and finally into osazones by the following important reactions :—

Aldose—

i. $-CHOHCH-\overset{\text{Aldose group}}{O}+H_2-\overset{\text{Phenyl-hydrazine}}{NNHC_6H_5}$

$$= -CHOHCH=N-NHC_6H_5 + H_2O$$

Hydrazone

ii. $-CHOHCH=N-NHC_6H_5 + C_6H_5NHNH_2$

$$= -COCH=N-NHC_6H_5 + C_6H_5NH_2 + NH_3$$

Carbonyl compound

iii. $-C\overset{\cdots}{-}O\overset{\cdots}{-}CH=N-NHC_6H_5 + C_6H_5NHN\overset{\cdots}{-}H_2$

$$= \begin{array}{l} -CCH=NNHC_6H_5 \\ \quad\| \\ \quad NNHC_6H_5 \end{array} + H_2O$$

Osazone

Ketose—

i. $-\underset{\text{Ketose group}}{COCH_2OH} + H_2NNHC_6H_5 = \begin{array}{l} -CCH_2OH \\ \quad\| \\ \quad NNHC_6H_5 \end{array} + H_2O$

Hydrazone

ii. $\begin{array}{l} -CCH_2OH \\ \quad\| \\ \quad NNHC_6H_5 \end{array} + C_6H_5NHNH_2$

$$= \begin{array}{l} -CCHO \\ \quad\| \\ \quad NNHC_6H_5 \end{array} + C_6H_5NH_2 + NH_3$$

Aldehyde compound

iii. $\begin{array}{l} -CCH\overset{\cdots}{-}O + H_2\overset{\cdots}{-}NNHC_6H_5 \\ \quad\| \\ \quad NNHC_6H_5 \end{array}$

$$= \begin{array}{l} -CCH=NNC_6H_5 \\ \quad\| \\ \quad NNHC_6H_5 \end{array} + H_2O$$

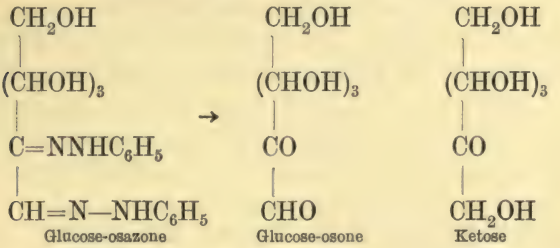
Osazone

In both cases it will be seen that the osazone grouping is the same whether derived from an aldose or a ketose ; if two sugars, therefore, yield different osazones it is a proof that they differ in constitution in portions of the molecule *other than the aldose or ketose group.*

As the osazones are mainly soluble, crystallisable compounds with definite melting-points, they are exceedingly useful both in isolating and identifying the various sugars.

Sugars can be obtained from osazones by the action of strong hydrochloric acid which eliminates the phenyl-hydra-

zine group, forming a ketone-aldehyde which on reduction yields a sugar. Thus in the case of glucose-osazone we have the following sequence of compounds :—



4. By successive treatment with hydrocyanic acid and hydrochloric acid, acids are formed as follows :—

$$R{-}CHO + HCN = R{-}CH\begin{matrix}OH\\CN\end{matrix}$$

$$R{-}CH\begin{matrix}OH\\CN\end{matrix} + 2H_2O = R{-}CHOHCO_2H + NH_3$$

On reduction by nascent hydrogen of the acid so formed, an aldose containing one more carbon atom than the sugar first taken is produced.

$$R{-}CHOHCOOH + H_2 = R{-}CHOHCHO + H_2O$$

It will be noted that an additional carbon atom is in this way attached to the chain, and so a means is afforded of producing a series of sugars, each member of which contains one carbon atom more than the preceding one. By this method the sugars above referred to containing seven, eight, and nine carbon atoms have been produced. This reaction, which is of great importance, was discovered by Kiliani, whose name it bears.

5. Inasmuch as the aldoses and ketoses are capable of oxidation, they themselves act as reducing agents, and so they

are capable of reducing certain metallic salts with production of the metal or a lower oxide of the metal. Thus, an ammoniacal solution of nitrate of silver when warmed with ordinary glucose (grape sugar) yields a brilliant mirror of silver.

On warming with an alkaline solution of copper potassium tartrate (known as Fehling solution) a red precipitate of cuprous oxide, Cu_2O, is produced. This is an important reaction which can be used for the quantitative determination of the amount of reducing sugar present in a solution.

Stereo-isomerism of the Ketoses and Aldoses.—If the formula for an aldose or ketose containing more than two carbon atoms be carefully studied, it will be seen that in most cases one or more asymmetric carbon atoms are present in the molecule. Thus to take the simplest case, viz., the aldose form of glycerose CH_2OH **C** HOHCHO, the centre carbon atom is combined with four different atoms or groups, and consequently a right-hand and left-hand and also an inactive form of this sugar are capable of existence.

In the case of a hexose the number of asymmetric carbon atoms, and consequently of right-hand and left-hand forms, becomes considerable ; thus a ketohexose contains three asymmetric carbon atoms :—

$$CH_2OH\ \mathbf{C}\ HOH\ \mathbf{C}\ HOH\ \mathbf{C}\ HOHCOCH_2OH$$

An aldohexose contains four asymmetric carbon atoms :—

$$CH_2OH\ \mathbf{C}\ HOH\ \mathbf{C}\ HOH\ \mathbf{C}\ HOH\ \mathbf{C}\ HOH$$

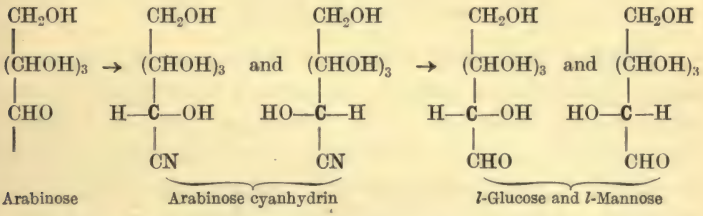
The separation and identification of the large number of possible ketohexoses and aldohexoses is a very complicated task ; mainly by the exertions of Emil Fischer and his pupils it has to a great extent been accomplished.

It would lead too far, and would be foreign to the subject of the present work, to consider the methods of preparation and identification of all these compounds in detail. It may be

stated generally that the researches have been conducted on the following lines :—

(*a*) The production of aldoses or ketoses from naturally occurring substances whose ordinary constitutional formulæ and specific optical activity are known. Thus dextro mannite or mannitol, which can be readily obtained from naturally occurring manna, yields dextro mannose on oxidation.

(*b*) The building up of sugars from compounds of known constitution by Kiliani's reactions. Thus arabinose yields eventually *l*-glucose or *l*-mannose as follows :—



(*c*) Passing from ketose to aldose or *vice versâ* by means of the phenyl-hydrazine compounds; the example already given on p. 87 will illustrate this.

(*d*) Resolving inactive compounds by fractional crystallisation of suitable salts, or by the action of enzymes.

It will be useful briefly to describe the chief properties of one or two well-known members of the hexose group and of certain related compounds, which are of interest from the fact that they have been used as a means of differentiating certain bacteria one from another, by the capacity or otherwise which these may possess of fermenting the substance in question.

Mannite or Mannitol has already been referred to; it is a 6 carbon alcohol of the general formula $CH_2OH(CHOH)_4CH_2OH$. It occurs in manna, which consists of the evaporated sap exuding from various species of ash cultivated in southern Europe; it also occurs widely distributed in the vegetable

kingdom, e.g., in the roots of celery, in the sugar cane and in various algae and fungoid growths ; it can be extracted from manna by boiling out with dilute alcohol and recrystallising. It has a pleasant sweet taste, and is sparingly soluble in cold but readily in hot water.

Dulcite or Dulcitol is isomeric with mannite ; it occurs in Madagascar manna, from which it can be extracted by hot water. Dulcite is not so sweet tasting as manna and is less soluble in water. It is important to note that both mannite and dulcite yield secondary hexyl iodide, $CH_3(CH_2)_3CHICH_3$, when treated with concentrated hydriodic acid.

Glucose, also known as *dextrose* or grape sugar, is found in large quantities in grapes. As already mentioned, it is a characteristic member of the aldohexose group ; it occurs frequently, together with **lævulose,** also called *fructose* or fruit sugar, which is the corresponding ketohexose (see pp. 84, 85), in the juice of sweet fruits and in honey. The mixture of the two, dextrose and lævulose, is generally known as *invert sugar.* Dextrose and lævulose can be obtained from invert sugar by the crystallisation of the dextrose from an alcoholic solution ; or by the preparation of an insoluble lime compound of lævulose, which is decomposed by suspending it in water and passing carbon dioxide through the mixture. On filtering off the calcium carbonate and evaporating the filtrate the lævulose is obtained as a syrup which can be crystallised from alcohol.

Inosite.—This is a somewhat rare sugar which is obtained as an extract from the heart or lungs of the ox by a complicated process ; it crystallises from dilute alcohol with two molecules of water.

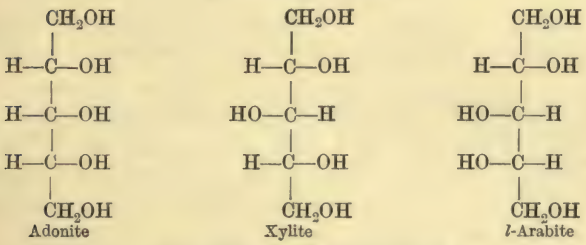
Galactose is a sugar formed along with dextrose when milk sugar is boiled with dilute sulphuric acid ; it is also formed when gum arabic is similarly treated. It is less soluble

than dextrose and can therefore be separated from it by crystallisation.

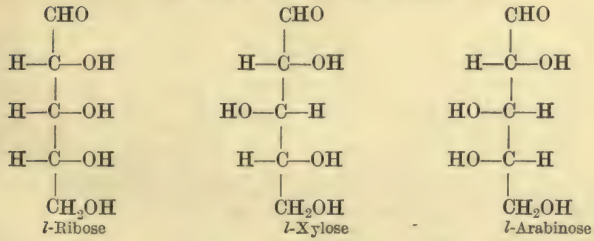
The relationship of these and other related sugars, together with their corresponding alcohols, will be rendered clear from the following Table II, based on the researches of Emil Fischer and other workers :—

TABLE II

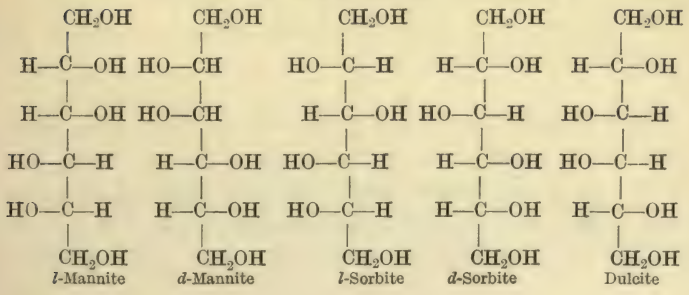
Five-carbon Alcohols



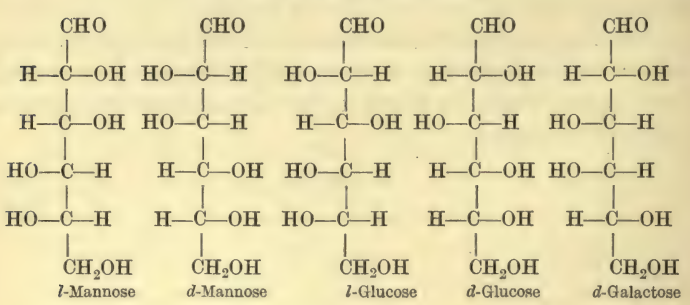
Five-carbon Sugars (aldopentoses)



Six-carbon Alcohols



Six-carbon Sugars (*aldohexoses*)



The Di-saccharoses.—The chief members of this group are *cane sugar*, *milk sugar*, and *malt sugar*. By the action of dilute acids, or, as will be shown later, by the activity of certain organisms or enzymes, they are converted into hexoses according to the general equation

$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6$$

This splitting up of the di-hexose into two hexoses is generally known as *inversion*, because in the case of cane sugar which is dextro-rotatory, the resultant mixture of sugars is lævo-rotatory, owing to the fact that the lævo-rotatory power of fructose or lævulose is greater than the dextro-rotatory power of glucose or dextrose.

Cane sugar forms glucose and fructose, lactose forms glucose and galactose, and maltose two molecules of glucose.

The following are a few interesting facts in regard to the three sugars above mentioned :—

Cane Sugar.—Saccharose or sucrose occurs in large quantities in the sugar cane and in beetroot, from which two sources the world derives practically the whole of its sugar. Both in the case of sugar cane and beetroot, the general methods of extraction are much the same. The material

is either crushed in presses and the juice thus forced out, or the sugar is systematically extracted by water; the extract is clarified with lime, decolourised with animal charcoal, filtered and evaporated in vacuum pans till the sugar crystallises. The dark mother liquor is known as *molasses* or treacle, the sugar can be obtained from it by precipitation with strontium hydroxide; from this precipitate the sugar is recovered by suspending in water, passing carbon dioxide through, filtering from the strontium carbonate and evaporating.

Cane sugar crystallises from water in hard four-sided prisms; it is generally purified for purposes of scientific investigation by recrystallisation from hot alcohol. It melts at about 160° C. and is dextro-rotatory; it does not reduce Fehling solution; it also does not combine with phenyl-hydrazine.

Maltose is obtained from starch by the action of the enzyme known as amylase; this reaction forms the subject of Chapters V and VI of this book.

Maltose is much more soluble in water than is cane sugar, and is more strongly dextro-rotatory; it also reduces Fehling solution and combines with phenyl hydrazine. A further distinction from cane sugar lies in the fact that it readily ferments with yeast, which is not the case with cane sugar.

Milk sugar or lactose occurs in the milk of all mammals to the extent of about 4 per cent.

In the manufacture of cheese, milk is treated with a clotting enzyme known as 'rennet' which coagulates the casein, milk sugar remaining in solution; it can be readily crystallised from this solution on evaporation, the crystals containing one molecule of water of crystallisation. It is much less sweet than cane sugar and is dextro-rotatory, though to a less extent than cane sugar; it reduces Fehling solution slowly on boiling, but like cane sugar it does not

ferment with pure yeast, nor does it form a phenyl-hydrazine compound.

The Poly-saccharoses.—Of these, *starch* and *cellulose* will be more usefully considered separately in the chapters devoted to their decomposition by enzyme or bacterial action. It will be understood that their molecular structure is much more complicated than that of the carbohydrates belonging to the two preceding classes.

Glucosides.—A class of substance occurs in nature, generally in the leaves of plants or bark of trees, which on treatment with acid, or by the action of certain enzymes, yields a sugar together with another organic compound. In the majority of cases the sugar present is glucose, and these bodies, therefore, are termed *glucosides*.

One of the earliest and, at the same time, best known of the glucosides is *amygdalin*, which occurs in bitter almonds and in the kernels of apricots, peaches and plums.

Liebig and Wöhler in 1837 isolated an enzyme which they termed *emulsin*. They found that on crushing bitter almonds the amygdalin was decomposed according to the following equation :—

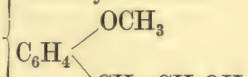
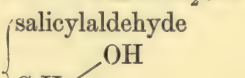
$$\underset{\text{Amygdalin}}{C_{20}H_{27}O_{11}N} + 2H_2O = \underset{\text{Benzaldehyde}}{C_7H_6O} + \underset{\text{Hydrogen cyanide}}{HCN} + 2\underset{\text{Glucose}}{C_6H_{12}O_6}$$

Recent researches by Fischer, Caldwell and Courtauld and others have shown that amygdalin can be split up at several centres marked *x y z* in the formula

$$\underset{z}{NC} \cdot CHC_6H_5 \cdot \underset{x}{O} \cdot C_6H_{10}O_4 \cdot \underset{y}{O} \cdot C_6H_{11}O_5$$

which are attackable only by specific enzymes; thus an infusion of yeast only splits off one molecule of grape sugar at *y*, leaving a residue termed *almond nitril glucoside* which is capable of being completely split up by emulsin.

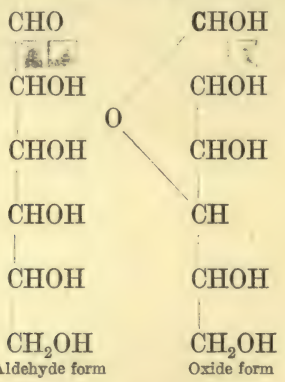
The following are a few typical naturally occurring glucosides, together with their products of hydrolysis :—

Arbutin $C_{12}H_{16}O_7$	yields glucose *plus*	hydroquinone $C_6H_4(OH)_2$
Salicin $C_{13}H_{18}O_7$	" " "	salicyl alcohol $C_6H_4OHCH_2OH$
Coniferin $C_{16}H_{22}O_8$	" " "	coniferyl alcohol 
Helicin $C_{13}H_{16}O_7$	" " "	salicylaldehyde 
Indican $C_{14}H_{17}O_6N$	" " "	indoxyl C_8H_7ON
Sinigrin $C_{10}H_{16}O_9NS_2K$	" " "	allyl mustard oil C_3H_5CNS + $KHSO_4$

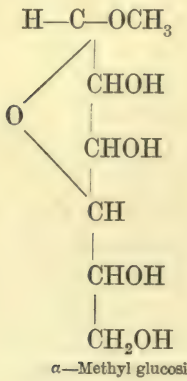
The naturally occurring glucosides are accompanied, in most cases at any rate, by the enzyme which is capable of effecting their hydrolysis. The enzyme and the glucoside occur in separate cells, and they only act upon one another when the cell contents are brought together by crushing, as for instance, when bitter almonds are pounded in a mortar; the emulsin in this case is probably contained in the skin of the almond. It is possible that glucosides form a reserve of food material for the plant, their constituents being only capable of assimilation after hydrolysis, that is, when brought in contact with the enzyme.

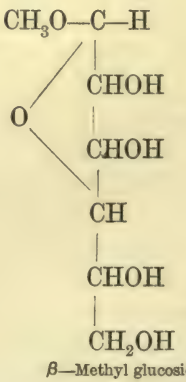
It has been possible to prepare a certain number of substances artificially which belong to the same class as glucosides. When glucose is dissolved in water and the freshly prepared solution examined in the polarimeter, the optical activity observed immediately after solution is found gradually to diminish, and after about six hours becomes constant at a

value rather less than half that of the original solution. It has been concluded that the glucose molecules when in solution exist both in an aldehyde form and in an oxide form, viz. :—



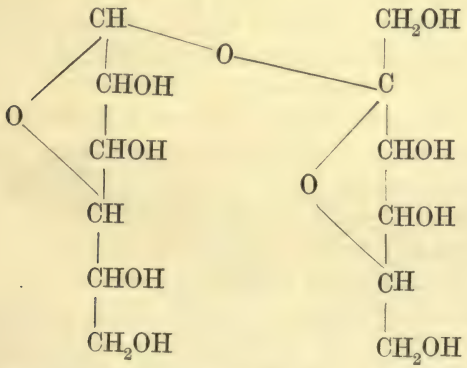
It will be seen that in the oxide formula the terminal carbon atom attached to the oxygen is asymmetric, and consequently two different derivatives are possible. By acting upon glucose with methyl alcohol in presence of hydrochloric acid two methyl esters have been obtained, which are really the simplest members of the glucosides, their formulæ being as follows :—





A number of similar compounds have been prepared by Emil Fischer, and it has been found by him and by E. F. Armstrong and others that the α and β glucosides show well-defined differences in their resistance to the action of enzymes. It has been further found that if the hexose resulting from decomposition of the glucoside is added to the reacting mixture of glucoside and enzyme the action is retarded. These investigations suggest that the decomposition of glucosides which is effected by enzymes is first preceded by a combination of the glucoside with the specific enzyme. They would indicate that enzymes are also asymmetric products, and in the words of Emil Fischer : ' Enzyme and glucoside must fit each other like key and lock in order that the one may exercise a chemical action on the other.'

The di-saccharoses have probably a glucosidic structure, saccharose or cane sugar being the glucoside of glucose, with the following probable formula :—



The enzymic hydrolysis of the di-saccharoses and poly-saccharoses is of great technical and scientific importance and will be dealt with in separate chapters in the following pages.

CHAPTER V

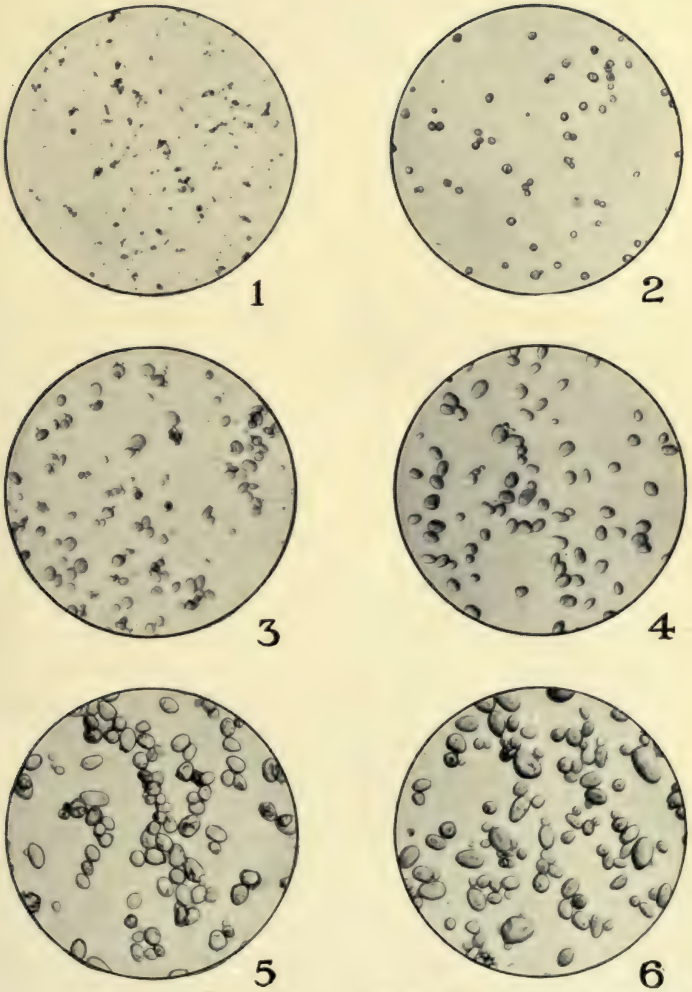
THE HYDROLYSIS OF STARCH BY AMYLASE

STARCH occurs widely in the vegetable world, being the first visible product of assimilation in plants containing chlorophyll. Starch is usually manufactured in Europe, from wheat, maize, rice and potatoes; and in tropical countries, from the palm and from tubers of various plants.

Examined under the microscope, starch, which in the mass is a white powder, is seen to consist of small granules which have the power of polarising light. Different species of starch vary greatly in the size of their granules. This is clearly seen in Plate I, reproduced from actual photographs, the same magnification being used in every case. Under high magnification, especially after treatment with dilute alkali, the starch granules can be seen to consist of a series of layers arranged round a nucleus.

Starch is found to consist of several isomeric compounds, the chief portion being starch proper, termed either amylum or granulose, the remainder consisting of starch cellulose or farinose. The starch cellulose is not readily attacked by enzyme action or by acids. Soluble starch consists of granulose from which the less soluble starch cellulose has been removed.

If a solution of starch is boiled for some time with dilute acid the solution will become clear and it will be possible by suitable tests to recognise the presence of a sugar in the solution. The same reaction is brought about if a small



[*Photo by Messrs. Flatters & Garnett, Ltd.*

PLATE I.—STARCHES (Magnification × 70 diam.).

1. Rice. 2. Maize. 3. Wheat.
4. Arrowroot. 5. Sago. 6. Potato.

amount of extract of malt is added to the starch solution. This reaction, which is known as the hydrolysis or saccharification of starch, has been found to be due to an enzyme which has been termed **amylase.** Before studying the reaction further it will be useful briefly to describe the characteristics and method of preparation of malt. **Malt** is barley which has been allowed to germinate up to a certain point, after which the process is arrested by heat.

The processes in the manufacture of malt are as follows :—

(1) *Preliminary cleaning, grading, etc.*—This is effected in ordinary screening and winnowing apparatus, much dust and dirt having frequently to be removed from certain classes of barley, especially those sent from the East.

(2) *Steeping.*—The clean barley grains are steeped in water in vats until quite soft.

(3) *Malting.*—The steeped grains are placed on floors and constantly turned over until they begin to germinate.

(4) When *germination* has gone far enough the grains are placed on drying floors and finally heated in kilns to a temperature not exceeding 180° F. This process has to be carefully conducted and lasts some days ; the malt is then screened to remove the dry rootlets and finally stored.

Malt will be seen on inspection to differ from barley in the following particulars : The barley grain is hard and difficult to break with the teeth and has no special taste or smell ; the malt, on the other hand, is friable, has a pleasant odour and sweet taste. On separating the barley grain, especially if it has been previously soaked in water, the germ will be readily distinguished at the base of the grain (cf. Plate II). In the case of the malt the germ will be found to have developed some two-thirds of the length of the grain. It is now known as the *acrospire.* The precise change taking place in the barley grain during the process of malting will be more fully studied in the next chapter ; for the moment it will suffice that in malt we have a substance containing a store of amylase

which will enable us to study the action of this enzyme upon starch and thereby to obtain a knowledge of the conditions of enzyme action in general.

In the first place it will be necessary to prepare some malt extract, and it may here at once be stated that it is of fundamental importance in all work connected with the preparation and study of enzymes that the conditions of experiment should be very carefully under control, more especially the temperature. For this purpose constant-temperature incubators (see Fig. 7) are essential. It is also better to use constant temperature water-baths; Fig. 4 *b* shows a convenient type of water-bath for this kind of work. Small beakers capable of holding conveniently about 100 c.c. of solution can be fitted neatly into this bath by means of flat rings; test-tubes can also be held in position, or stacked in the beakers.

To prepare a cold water extract of malt 100 grams of ground malt are mixed with 250 c.c. of water and the mixture allowed to stand with frequent stirring for about five hours. For the purpose of grinding the malt a small hand-mill similar to a coffee grinding mill can be used.

The various starches differ considerably in the ease with which they are attacked by amylase, and for the purpose of experiment it is better to use so-called *soluble starch*, which is prepared after the manner described below.[1]

[1] Cf. Brown, *Laboratory Studies*, p. 65. *Preparation of Soluble Starch.*—Introduce about fifty grams. of potato starch into a 500 c.c. flask, and half fill the flask with a 7·5 per cent. solution of hydrochloric acid made by diluting 125 c.c. of the concentrated acid to 500 c.c. with distilled water. Allow the starch to digest with the dilute acid at the ordinary room temperature for seven or eight days. The acid should then be poured off and the starch washed repeatedly with distilled water by decantation until the *granules* no longer give an acid reaction when placed on blue litmus-paper. One or two drops of dilute ammonia should then be added, and the starch again washed until every trace of ammonia is removed. Drain the starch thoroughly on a filter, and spread it on filter-paper to air-dry at a temperature of about 25° C. (77° F.).

The action of malt extract upon starch may now be studied as follows: A 3 per cent. solution of starch paste (i.e., six grams of starch to 200 c.c. of water) is first prepared. In preparing starch paste the starch should first be rubbed down in a mortar to a thin cream with a portion of the water used, while the remaining volume of water is heated to boiling; the starch cream is then carefully added, stirring the while. A solution of soluble starch prepared as above contains no visible undissolved particles.

With the extract of malt and the cold starch solution it is now possible qualitatively to examine the changes which occur when the two are brought together. For this purpose, say, six small beakers or large test-tubes may be used and about 10 c.c. of starch solution placed in each, together with $\frac{1}{2}$ c.c. of the filtered malt extract. The test-tubes are then placed in a constant-temperature water-bath at a temperature of 60° C. The test-tubes can now be observed from time to time. The first change to be noted is the clarification of the starch; simultaneously with this it may be found that the ordinary blue colour is no longer given when a drop of dilute iodine solution is added to the solution. On removing the second test-tube after the lapse of a further period, the colour of the iodine will be found to have become distinctly red. If simultaneous tests are made by the addition of Fehling solution, a gradually increasing amount of precipitation will be noted until finally no reaction is given by the iodine, while a copious red precipitate is formed on boiling with the Fehling solution.

This experiment indicates that the action of the malt extract upon the starch solution is progressive. In the first place a substance is formed which gives a purple colour with iodine but does not reduce Fehling solution. Later on substances are formed which give a red coloration with iodine, and eventually only the sugar or Fehling reducing substance can be detected. A more exact investigation of the first

change will be attempted later; it is sufficient here to say that the substances which give colour reactions with iodine are known as *dextrins*, owing to their effect on polarised light; the sugar can be shown to be maltose.

The nature of the substance present in the *malt extract* which brings about the change has now to be considered. If the extract is added to alcohol, a white precipitate is formed. This precipitate can be shown to contain the active substance or enzyme in question, which, as it is concerned in the breaking down of starch, is known as **amylase** (or frequently 'diastase'). Its preparation and investigation will illustrate very well the properties and method of preparation of enzymes in general.

The following method may be used for the preparation of the amylase of malt: 100 grams of ground malt (preferably air dried) are digested with 250 c.c. of 20 per cent. alcohol for four hours and then filtered. Strong alcohol is added to the filtrate so long as a white flocculent precipitate is formed; this precipitate contains the amylase, it is allowed to stand and the supernatant liquid poured off. The precipitate is washed by decantation with a little strong alcohol, and afterwards transferred to a smooth hardened filter and washed repeatedly with small quantities of absolute alcohol. Portions of the precipitate may now be examined in various ways. By warming as much as will go on the end of a knife blade with about 20 c.c. of starch solution the above described changes in the starch solution will be found to take place. The following reactions are characteristic of enzymes in general:—

1. A small portion of the precipitate is dissolved in the least possible quantity of water and a few drops of an alcoholic solution of guiachum resin are added, together with a little hydrogen peroxide. An intense greenish blue colour is obtained. By taking different quantities of the aqueous solution it will be found that the blue colour varies in proportion to the con-

centration ; in this way the actual amount of enzyme present in the solution may be roughly determined. This method is very useful for quickly following the rate at which an enzyme is developed under different conditions, e.g., at the different stages of growth of the roots or leaves of plants, or during the progress of a technical process, e.g., the withering of tea leaves.

2. A small portion of the precipitate is warmed with strong caustic soda ; the presence of ammonia can be recognised by the smell and by introducing red litmus paper into the upper portion of the test-tube.

3. A small portion of the precipitate is dissolved in strong caustic soda and a few drops of very dilute copper sulphate solution added ; a violet colour is produced. This is known as the *biuret* reaction, as it is given by biuret, a substance produced by heating urea.

4. To the aqueous solution of a portion of the precipitate a few drops of *Millon's reagent* are added ; a white precipitate is obtained. Millon's reagent is a solution of mercuric nitrate containing free nitric acid. It is prepared by dissolving one part of mercury in two parts of strong nitric acid and diluting the solution with twice its bulk of water ; after standing some time the supernatant liquid is decanted from the precipitate.

5. A portion of the precipitate is warmed in a small porcelain dish with a little concentrated nitric acid, and the excess of acid gently evaporated ; on addition of a drop or two of strong ammonia a bright orange colour is obtained. This is known as the *Xanthoproteic* reaction.

All the above reactions, with the exception of the colour reaction with guiachum resin and hydrogen peroxide, are characteristic of albumin and its derivatives. Enzymes, therefore, can be broadly described chemically as complex nitrogenous substances akin to albumin. Many attempts have been made to obtain enzymes in the pure state, but with little success. Like all complicated nitrogenous bodies of

this class they tend to carry down with them other substances which are present in solution, especially inorganic salts ; it is therefore very difficult to obtain them free from ash. Moreover, in the course of the operations necessary to prepare them in an approximately pure state, they tend to suffer a loss in activity. So difficult indeed is it to obtain them as definite chemical compounds that it has been seriously suggested that enzyme action is really a property of matter, such, for example, as radio-activity or static electrical potential.

The following method will, however, serve to illustrate the preparation of amylase in an approximately pure condition.

The precipitate, formed as already described by adding the malt extract to alcohol, contains, besides the active enzyme, a quantity of carbohydrate (dextrin and sugar) together with albuminoid matter and salts. It is possible to eliminate the carbohydrate and the albuminoid impurity to a large extent by the action of yeast, if the latter has been previously starved of nitrogen, by allowing it to remain for twenty-four hours in a 10 per cent. solution of sugar. To prepare the amylase in this way, 100 grams of crushed malt are macerated with 300 c.c. of water at a temperature of 30° C. for eighteen hours, stirring at half-hour intervals. The mass is filtered and pressed and thoroughly washed with water, the washings being mixed with the original extract. After filtering the solution is made up to 300 c.c. with water, ten grams of beer yeast added and left at a temperature of 28° C. for forty-eight hours. The solution is then filtered and 700 c.c. of alcohol added to the clear liquid ; the precipitated amylase is filtered through a hardened filter paper, washed with small quantities of absolute alcohol, and finally dried in a vacuum desiccator. About three grams of a white powder are obtained which has about 80 per cent. of the activity of the original extract.

A product of diminished purity and activity can be obtained if the treatment with yeast is omitted and the precipitate

with alcohol simply filtered, re-dissolved in water, re-precipitated with alcohol, washed with alcohol and dried *in vacuo*.

THE QUANTITATIVE STUDY OF THE ACTION OF AMYLASE ON STARCH

It is evident from the foregoing experiments that, to follow exactly the course of the change which takes place when malt extract acts upon a solution of starch, it is necessary to make use of methods which will enable the change to be followed when all the bodies concerned are present in solution together, since to isolate any one of them will be likely to decompose the others. The following properties are therefore made use of in studying the reaction :—

(1) Specific gravity ;
(2) Optical activity ;
(3) Cupric oxide reducing power.

(1) **Specific Gravity.**—It is possible to determine, e.g., the amount of sugar present in a solution by comparing the specific gravity of the solution with that of a solution of sugar of known strength.

The specific gravity is best determined by means of the specific gravity bottle. For this purpose a 50 c.c. specific gravity bottle with a perforated stopper is required ; the bottle must be cleaned thoroughly by washing with distilled water and rinsing out with a little strong alcohol. The bottle is then gently warmed over a flame and air sucked through by means of a glass tube or blown through with the foot-bellows until it is quite dry ; it is then allowed to cool in a desiccator and accurately weighed. The bottle is now filled with distilled water at a temperature of 15·5° C. (which is the temperature of graduation of the bottle) by completely immersing it in a beaker of distilled water which has been

carefully brought to this temperature in a constant temperature water-bath. The stopper is inserted, care being taken that no air bubbles are enclosed. The bottle is allowed to cool somewhat and then quickly wiped dry with a soft cloth and immediately weighed. Consecutive weighings in this manner should not differ by more than a milligram.

It is probable that the weight of water will not be exactly fifty grams, but the specific gravity of any liquid can be determined by filling the bottle with the liquid in a similar manner, weighing and dividing the weight of water in the bottle into the weight of the liquid.

In working with solutions of sugar or similar bodies, in order to determine the amount of sugar present from the specific gravity, a factor known as the *solution factor* is made use of.

Thus it has been found that ten grams of maltose made up to 100 c.c. at 60° F. has a specific gravity of 1038·5.

The amount of maltose contained in 100 c.c. of specific gravity 1055 will be $\frac{1055 - 1000}{3{\cdot}85} = 14{\cdot}285$ grams. The number 3·85 is termed the solution factor for maltose; dextrin has the same factor.

The specific gravity and, consequently, the solution factor are not the same for every carbohydrate, and an allowance must be made for this in specific cases.

(2) **Optical Activity.**—The subject of optical activity has been already dealt with in a general manner in Chapter IV.

The polariscope of Fig. 13 is adjusted as follows [1]: When the apparatus is well illuminated by the sodium flame, the zero position (the starting point of all experiments) must first be found: this is indicated by the two halves of the

[1] Based, by permission from Messrs. Baird and Tatlock (London), Ltd., on the instructions issued for use with the Lippich model half-shadow polariscope.

field appearing equally illumined (equal half-shadows). For this purpose the telescope F is focussed on the quartz plate,[1] so that the field presents a perfectly clear round circle divided into two equal parts by a sharply defined vertical line. If the graduated dial is turned through three or four degrees to either the right or the left of the zero line, it will be seen that one half of the field will become lighter, the other half darker.

In the first place, the zero position is so adjusted that the zero line of the circle coincides with the zero line of the vernier. The half-shadow can now be made lighter or darker (according as the polariser is turned to the right or left of the zero line) by means of the pointer reaching from the dial segment. When the pointer *h* is in the zero position, and at the same time the analyser A is placed in the zero position, both halves of the field of view appear black. The nearer the pointer is to the zero line, the darker the half-shadow will become, and the more sensitive the apparatus; but when the solutions are not quite transparent, the pointer must be moved more or less away from the zero line, so that the field is clear. For the majority of experiments the position of the pointer at $7\frac{1}{2}°$ is most suitable, therefore the apparatus is usually so adjusted that in this position the dial and vernier read exactly 0. When the pointer is moved, of course the zero point of the apparatus changes, and no longer corresponds with the zero line of the dial. The difference between the latter and the zero position of the apparatus must either be taken into account (the simpler way), or else after the graduated dial has been moved to 0 the apparatus must be again placed in the zero position: to do this, the analysing Nicol prism is turned, by means of the screws A, to the right or left until the half-shadows are equal in tint.

Special attention must be called to the following circumstance, which, if not noted, may lead to considerable confusion.

When the circle has been turned too far, and has gone

[1] Or small Nicol prism in the Lippich model.

beyond the sensitive range of the apparatus, the light, on comparison, appears to a certain extent of the same intensity on either side of the vertical line, and this point may be mistaken for the zero position. Under these circumstances, even if the circle is turned through ten, fifteen, or even a greater number of degrees, hardly any change will be observed.

It is a matter, therefore, of the greatest importance, particularly after the sample to be examined has been placed in the apparatus, to see that when the circle has been turned a few degrees on either side of the zero line, the transition from light to shade, and *vice versâ*, is instantaneous.

On placing the sample to be tested within the apparatus, the first thing to do is accurately to adjust the telescope so that the field is quite clear and equally divided by the vertical line; then the circle is turned until the shades are exactly of the same intensity on either side of the line.

The angle rotated by a column of 10 per cent. solution of a sugar ten decimetres long is known as the *specific rotatory power* of the sugar.

In the case of a 10 per cent. solution of pure cane sugar ten decimetres long the angle is 66·5 degrees when sodium light is used; this is generally known as $[a]_D$. In practice it is convenient to use tubes one or two decimetres long.

If the quantity of sugar per 100 c.c. is known, the specific rotatory power is given in the following equation: When R = the reading of the polarimeter, L the length of tube, and C the number of grams per 100 c.c., then

$$[a]_D = \frac{R}{L \times \frac{C}{100}}$$

On the other hand, if the specific rotatory power of the solution is known, the quantity present can be calculated from a determination of the specific rotatory power, thus :—

$$\frac{C}{100} = \frac{R}{L \times [a]_D}$$

Further, if the weight of original substance present, e.g., starch, is known, and the specific rotatory power of dextrin and maltose respectively, then the amount of conversion which has taken place after the first appearance of dextrin can be determined by an observation of the specific rotatory power of the mixture, e.g., the specific rotatory power of dextrin is 195, of maltose 135·4. If the specific rotatory power of the mixture is, say 165·2, the relative proportions present can be calculated from the following equation :—

$$195x + 135{\cdot}4\,(1 - x) = 165{\cdot}2$$

x in this case will be found to equal 0·5, i.e., the dextrin and maltose were present in equal quantities.

(3) **Cupric Oxide reducing Power.**—The cupric oxide reducing power of sugars is conveniently referred to a typical sugar taken as a standard. This standard is generally known as K, the amount of CuO reduced by one gram of glucose being taken as 100.

The actual amount of CuO reduced from Fehling solution by one gram of glucose is 2·205 grams ; the sugar reducing say 1·345 grams CuO per gram would give the value for K as 61, thus :—

$$2{\cdot}205 : 1{\cdot}345 :: 100 : 61$$

In the case of starch it is usual to take maltose as a standard, in which case the letter R is used instead of K ; thus a substance with three-quarters the reducing power of maltose would be considered to have a reducing power R_{75} instead of $K_{45{\cdot}75}$. A rapid method for determining cupric oxide reducing power is given in the following paragraphs, based on the Report of the Malt Analysis Committee to the Council of the Institute of Brewing.

The method is there used for determining the diastatic activity of malt. It may be used generally for determining

the amount of copper oxide reducing sugar present in any solution.

Briefly, the process consists in adding successive small measured quantities of the sugar solution to a given volume of Fehling solution till complete reduction takes place, the end of the reaction being determined by means of a special indicator.

The Fehling solution is prepared as follows :—

(*a*) *Copper Solution.*—Recrystallised copper sulphate (69·2 grams) is dissolved in water and the solution made up to one litre at 60° F. with distilled water.

(*b*) *Alkaline Tartrate Solution.*—Rochelle salt, i.e., sodium potassium tartrate (346 grams) and caustic soda (130 grams) are dissolved in about 600 c.c. of distilled water, the solution cooled and made up to one litre at 60° F. with distilled water.

The two solutions are to be kept separate, and equal volumes mixed for each day's work, from which mixture the volumes specified in the analytical method are measured out at 60° F.

Preparation of the Indicator.—One gram of ferrous ammonium sulphate and the same quantity of ammonium thiocyanate are dissolved in 10 c.c. of water at a moderate temperature, say 120° F., and immediately cooled ; 5 c.c. of concentrated hydrochloric acid are then added. The solution so obtained has invariably a brownish-red colour, due to the presence of ferric salt, which latter must be reduced. For this purpose zinc dust is the most satisfactory reagent to employ, and a mere trace is sufficient to decolourise the solution if pure reagents have been employed.

When kept for some hours, the indicator develops the red coloration by atmospheric oxidation. It may, however, be decolourised by the addition of a further quantity of zinc dust, but its delicacy is decreased after it has been decolourised several times. For practical purposes the indicator may be

too delicate, and it is recommended to prepare it the day before it is required for use, as it gives the best results after the second decoloration.

The titration is carried out as follows :—

The Fehling solution must first be standardised by taking, say, 1 gram of pure dextrose, and dissolving in 200 c.c. of distilled water.

Five c.c. of the Fehling solution are accurately measured into a 150 c.c. boiling flask, and raised to boiling over a small naked Bunsen flame. The sugar solution obtained as above is added from a burette in small quantities at first of about 5 c.c., the mixture being kept rotated and boiled after each addition until reduction of the copper is complete, which is ascertained by rapidly withdrawing a drop of the liquid by a glass rod, and bringing it at once into contact with a drop of the indicator on a porcelain or opal slab.

The reduction is complete as soon as no red coloration, due to the formation of ferric thiocyanate, is produced.

Having once standardised the Fehling solution, the amount of reducing sugar present in any given solution can be simply determined, care being taken in the case of a solution containing an active enzyme to stop the action of the latter by addition of caustic soda (say 10 c.c. $\frac{N}{10}$ caustic soda to 100 c.c. of the solution), so that alteration in the composition of the solution may not take place in the course of the titration operations.

It is possible in this way to follow the course of change, say when starch solution is acted upon by amylase, by withdrawing portions of the solution from time to time, stopping the reaction with caustic soda and titrating as above.

The presence of maltose in the products of the action of amylase on starch can be demonstrated by the preparation of an osazone, by the reaction described on p. 87.

In order to prepare an osazone, to 0·01 gram of the sugar in about half a test-tube full of water, 0·1 gram of phenyl

hydrazine is added, together with 0·2 gram of sodium acetate ; the whole is warmed until solution takes place and then heated half an hour on a boiling water-bath.

Glucosazone formed in this way from glucose is almost insoluble in water, and has a melting-point 225° C. The osazone of maltose is soluble in 75 parts of water at 100° C., and its melting-point is 205° C. Maltose is further distinguished from glucose by its specific rotatory power, which is 140 degrees compared with 52·5. The cupric oxide reducing power is two-thirds that of glucose.

The methods of investigation which have just been described render it possible quantitatively to follow the changes taking place in the course of the action of amylase upon starch. A large number of investigators have published researches on this subject, the general result of which has been to lead to the conclusion that the starch molecule breaks down by a series of hydrations and subsequent decompositions, maltose being formed at each splitting, together with a dextrin of less molecular weight. Certain of these dextrins, as the qualitative examination of the reaction showed, give characteristic colour reactions with iodine, the red colour, e.g., being due to a dextrin termed *erythro-dextrin*.

Brown and Morris noted that when 80 per cent. of maltose and 20 per cent. of dextrin had been formed, the last 20 per cent. hydrolised with difficulty, and they assumed the formation of a body intermediate between maltose and dextrin which they termed malto-dextrin. Their theory to account for this assumes that the starch molecule breaks up into a stable dextrin and so-called amylin groups which are capable of gradual hydrolysis to maltose ; we have thus the following equations :—

$$\underset{\text{Starch}}{5[(C_{12}H_{20}O_{10})_{20}]} = \underset{\text{Stable dextrin}}{(C_{12}H_{20}O_{10})_{20}} + 4\,[\underset{\text{Amylin groups}}{(C_{12}H_{20}O_{10})_{20}}]$$

$$(C_{12}H_{20}O_{10})_{20} + H_2O = \begin{cases} (C_{12}H_{22}O_{11}) \\ (C_{12}H_{20}O_{10})_{19} \end{cases} \text{ etc., etc.}$$

$$\text{to } (C_{12}H_{20}O_{10})_{20} + 19\,H_2O = \begin{cases} (C_{12}H_{22}O_{11})_{19} \\ (C_{12}H_{20}O_{10}) \end{cases}$$

The Conditions of Action of Amylase.—The study of the conditions under which the characteristic activity of amylase is manifested will serve as an example for the mode of action of enzymes in general; in many respects the action of the enzyme resembles the activity of a living organism, e.g. :—

1. Enzymes are destroyed by heat.
2. They have an optimum temperature of reaction.
3. They are not exhausted by continuous activity.
4. They are greatly affected by alterations in the medium in which they act, e.g., by certain antiseptics and poisons.

The following experiments will serve to illustrate the above statements :—

Experiment.—Two lots of 20 c.c. each of 3 per cent. starch solution are taken; to one is added 1 c.c. of unboiled malt extract, to the other 1 c.c. of boiled extract, and the two solutions warmed to 50° C. On testing with iodine and Fehling solution saccharification will be found to have taken place in the case of the solution to which the unboiled extract was added, while no change takes place in the second mixture.

Experiment.—A number of test-tubes may be taken containing, say, 20 c.c. of 3 per cent. starch solution and 1 c.c. of malt extract, and kept for an equal time at different temperatures, say, the ordinary laboratory temperature, an incubator at 20° C. and water-baths at 50° to 80° C. respectively. At the end of, say, ten minutes all the solutions are quickly brought to the boiling-point and titrated with Fehling solution and ferrous sulphocyanate. It will be found that the greatest amount of sugar has been formed at 50° C. By making a larger number of similar trials at different temperatures the

exact optimum temperature for a given enzyme can be determined.

Experiment.—The following experiment was devised by Effront to show the continuous activity of amylase: 200 c.c. of starch paste are mixed with 3 c.c. of malt extract and left for four hours at 30° C. The liquid is now diluted with distilled water to a volume of 300 c.c., 100 c.c. of this mixture is mixed with a further 200 c.c. of starch solution and heated for one hour to 50° C.; call this solution A. A second 100 c.c. of the original mixture is taken and boiled and afterwards added to another 200 c.c. of starch solution, together with 1 c.c. of the original malt extract. This mixture is heated to 50° C. for one hour; it may be called solution B.

Upon titration with Fehling solution the two solutions A and B will be found to give practically identical results, which indicates that 100 c.c. of starch mixture will do as much work as 1 c.c. of fresh malt extract, that is, that the amylase is not exhausted by continuous activity.

Experiment.—Three lots of starch solution of 200 c.c. each are taken, to one of them 0·25 gram of potassium or ammonium alum is added, to the second a few drops of strong solution of potash, while the third is left without any addition. To each solution 1 c.c. of malt extract is added and the three solutions are warmed for one hour at 50° C. Upon titration with Fehling solution the greatest action will be found to have taken place in the solution to which the alum has been added, while the action has been practically inhibited by the potash.

Effront has studied the effect of a number of salts, such as phosphates and acetates and of organic bases, e.g., asparagin, upon the action of amylase and has found that in most cases a considerable acceleration of the action takes place. These results are of special interest in view of the effect of phosphates upon the fermentation brought about by the enzymes of yeast, which has been studied by Harden and his colleagues.

The above experiments show the analogy which exists between the action of enzymes and the action of organisms. They can, however, be differentiated by certain other properties. Thus some enzymes, e.g., invertase, will pass readily through a porous porcelain filter, which under similar conditions will retain all living organisms. Further, certain antiseptics which inhibit the action of micro-organisms are without effect on enzymes; among these the most frequently used are thymol, chloroform, and especially toluene.

It has also been found that when the amount of substance to be acted upon is large in proportion to the quantity of enzyme used, then the amount of reaction taking place is proportional to the quantity of enzyme present; this is known as the *law of proportionality*. The following experiments may serve to illustrate it :—

Two lots of 300 c.c. each of 3 per cent. starch solution are taken; to one is added $\frac{1}{2}$ c.c. of malt extract, to the other 2 c.c. of the same extract, and the two mixtures heated for a quarter of an hour at 50° C. Upon titration with Fehling solution it will be found that the greater amount of reaction has taken place in the solution to which the larger quantity of malt extract was added.

CHAPTER VI

THE CONDITIONS OF FORMATION OF AMYLASE IN THE LIVING CELL

It has been possible in the case of amylase more than with many other ferments carefully to study the conditions under which it is produced, and it is therefore instructive to repeat, in a simple way, some of the experiments which have been made and so to obtain an insight into the methods of research made use of in this class of study.

In the first place then, as the chief source of amylase so far considered is the malted barley grain, it will be well to study more carefully the structure of the barley grain, and note the difference between it in its original condition and after the process of germination or conversion into malt has taken place. In order to examine the barley grain microscopically it is necessary first to soften it by immersion in water, possibly for a day or two, until it can easily be cut through with a knife ; there is then no difficulty in separating the outer skin or husk and in dividing the two halves of the grain. At the base of the grain in the cleft of the two halves will be noted the *embryo*.

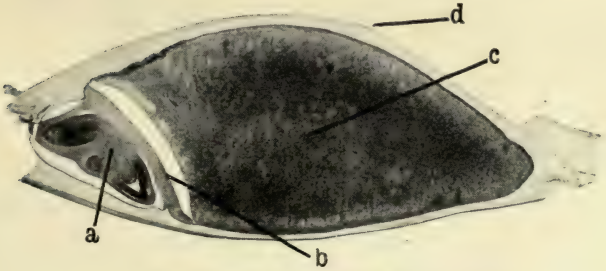
So much can readily be discerned by the naked eye. In order to obtain sections suitable for microscopical examination the following implements and reagents will be necessary :—

Some blocks of paraffin ;
A sharp razor or microtome ;
One or two mounted needles ;

PLATE II.



(i) Root Nodules of Pea.



(ii) Section of Barley Grain.

[*Photos by Messrs. Flatters & Garnett, Ltd.*

A few watch glasses ;
Absolute alcohol ;
Clove oil ;
Alcoholic iodine solution ;
Microscope slides and cover glasses ;
Xylol ;
Canada balsam ; and
Shellac varnish.

In order to make a section laterally through the embryo, a softened barley grain should be embedded laterally into a block of paraffin, say one inch cube, if the razor and not the microtome is to be used. The grain is easily embedded by melting a little of the paraffin in the middle of one of the sides of the block with a hot glass rod, carefully placing the grain in the little melted pool of paraffin and allowing it to set thoroughly hard. With a little practice it is possible with a sharp razor to cut very fairly accurate thin lateral sections of the grain ; a number of these can be cut until the embryo is fully exposed, when the section of the grain will have the appearance roughly as shown in Plate II (ii). A number of these sections should be cut and immersed in a little absolute alcohol, contained in one of the watch glasses, in order to harden. As the moisture in the section is reduced by alcohol the section becomes hard. It is next transferred to a watch glass containing clove oil, in order to clear it and render it transparent ; it is then immersed in xylol to remove the excess of clove oil, placed on a microscope slide, covered by a cover slip and examined. If a permanent preparation is required it may be dipped into an alcoholic solution of iodine and then into picric blue, and the excess of iodine and of colour washed out with alcohol. The iodine stains the starch granules purplish blue, and the aleurone (or albuminoid) layer yellow. The remaining tissues are coloured blue by picric blue.

The section is now ready for mounting ; it is placed in the centre of a microscope slide, covered with a drop of Canada

balsam and the cover glass pressed down over it, any excess of Canada balsam exuding from the edges of the cover glass being carefully wiped away with a clean rag. As soon as the Canada balsam is set, the section is ready for examination under the microscope.

Before making a permanent preparation it is well to examine a number of sections in order to obtain one which is really characteristic. A good section will show the structure of the grain as in Plate II (ii). Here *a* is the germ, *b* the scutellar epithelium which divides the germ from the endosperm *c*, while *d* is the husk.

If the section of a barley grain so obtained be compared with a section of undried malt, in the first place by simple examination of the grain with the naked eye, it will be seen that the germ has grown very considerably and that the cells of the endosperm are broken down, so that the main bulk of the grain is soft and friable, and it is extremely difficult to make a microscopic section of it in this condition. As a matter of fact the cellulose walls of the starch-containing cells have been broken down in the first stage by a cellulose dissolving enzyme, and afterwards the amylase has penetrated the bulk of the endosperm and has largely saccharified the starch present.

The difference in the distribution of amylase in the unmalted and malted barley grain can be seen if a section through the median line is treated with a small quantity of guiachum resin and hydrogen peroxide, when the blue colour will be found to extend all over the grain in the case of the malt, but to be only noticeable in the neighbourhood of the embryo in the case of the barley. This observation suggests that the seat of production of amylase is in the embryo; this can be proved by the following experiment first made by Brown and Morris.

Some starch gelatine is prepared by adding 7 grams of gelatine to 100 c.c. of a 1 per cent. solution of soluble starch in water, warming until the gelatine is uniformly dissolved and

sterilising in a steam steriliser. With this starch gelatine a number of cultivation tubes and plates may be prepared in order to determine the production of amylase under different conditions.

For the determination of the production of amylase by the growing embryo of the barley grain, a small deep Petri dish may be taken, and the starch gelatine poured in to the depth of about $\frac{1}{4}$ inch and allowed to set. By means of a sterile needle or knife blade an embryo may be detached from the grain, previously softened in water, and placed on the surface of the starch gelatine. It can be brought into close contact with the starch gelatine by melting a minute portion of the jelly immediately under the embryo with a warm sterile needle. Several embryos may thus be set up and allowed to remain at a temperature of about 18° C. for a day or two. At the end of that time sections of the jelly a little wider than the embryo may be cut out so that the jelly immediately below the embryo can be observed. On treating the slices of jelly with a little dilute iodine solution it will be found that a semicircular space below the embryo is colourless, thus showing that the embryo has secreted amylase, which has saccharified the starch in its immediate vicinity.

By making similar observations with the other embryos used, at intervals, say, of twenty-four hours, it will be seen that the area affected increases as the embryo develops.

Brown and Morris have shown that embryos separated from the barley grain in this way can be grown on quite a variety of different media. Thus, e.g., barley embryos could be grown in the endosperm of a wheat grain, the embryo of the latter being removed. They can also grow in solutions of sugar or even on moist filter paper, their action in the last two cases being very probably due to the secretion of enzymes other than amylase. Careful experiment has shown that the amylase is secreted mainly by cells in the neighbourhood of the *scutellar epithelium* (*b*, Plate II (ii)).

By means of further observations, using starch gelatine as a cultivation medium, it can be shown that various *micro-organisms* are capable of secreting amylase. Thus, e.g., an ordinary Petri dish may be taken and a thin layer of melted starch gelatine poured into it and allowed to set. A few drops of ordinary sewage diluted ten times with water can then be run over the surface of the jelly, any excess being poured off; at the end of twenty-four hours a number of colonies will probably have appeared. On pouring a dilute solution of iodine on and off the plate, a number of colonies will be found to be surrounded with white rings, showing that the starch has been saccharified in their immediate neighbourhood, i.e., that the particular organism forming the colony has the power of secreting amylase. It is possible, of course, to take out such colonies with a sterile platinum wire and prepare streak cultures in starch gelatine tubes.

In order to be sure that the white ring observable on addition of iodine is not simply due to the production of alkalinity in the medium at that point, the plate may be treated with dilute hydrochloric acid prior to the addition of iodine, but in this case there is danger that the colonies may be sterilised.

Among the bacteria which produce amylase Koch's cholera bacillus may be mentioned, also *B. anthracis*, *B. megatherium*, and *B. lactis aerogenes*, which is a characteristic sewage organism. *B. coli communis* does not, however, secrete amylase; in fact, this organism can be used as an elegant test for the production of sugar by an amylase-secreting organism, such as the bacillus of cholera or anthrax, by growing the latter in starch gelatine and then incubating with *B. coli*, when the characteristic gas formation due to the fermentation of sugar by this organism will be noted.

That the saccharification of the starch is really due to the formation of amylase by the organism, and that it is not due simply to its ordinary developmental activity, may be proved by taking a little of the converted starch gelatine, melting

with a little thymol in order to inhibit vital phenomena, and adding the mixture to a further quantity of starch gelatine, when saccharification will continue, showing that the change is due to an enzyme secreted by the organism which is capable of acting whether the organism be alive or not.

Besides numerous bacteria a certain number of moulds are also capable of secreting amylase, e.g., *Aspergillus niger*; this can readily be demonstrated by making a streak culture of this organism in a tube of starch gelatine. After some days, when a vigorous growth of the mould has taken place, the gelatine may be melted, dissolved in warm water, and filtered from the mould and the filtrate tested with Fehling's solution for the presence of maltose.

All the foregoing experiments necessitate care in manipulation in order to prevent infection by extraneous organisms, but with a little practice in bacteriological technique they are not difficult to carry out and are highly instructive. The secretion of an enzyme, such as amylase, is analogous to the secretion of toxins by pathogenic organisms, and the chemical problems involved in all these cases are of a similar nature. It will be shown later that certain organisms, e.g., *Aspergillus niger*, are capable of secreting enzymes suitable to the conditions of their environment. Thus, e.g., *Aspergillus niger*, is capable, not only of saccharifying starch, but also of inverting cane sugar and of splitting up fats; in fact, it has been shown by Delépine that this organism can derive sustenance from almost every conceivable organic medium. Similarly, a yeast cell can bring about quite a number of different chemical changes. Organisms of simpler structure and function, such as bacteria, are more limited in their range of activity, but there is no doubt that they too are capable of bringing about a variety of changes according to their differing environments. A possible explanation suggests itself here of the difference in pathogenic effect, which is observed when the same organism is cultivated under differing conditions.

There is evidence also that the amylase secreted, e.g., by the growing plant embryo and by growing micro-organisms, is somewhat different chemically from the amylase secreted by the purely vegetative organs of plants, e.g., the leaf cells, and by animal cells. Thus, an amylase is secreted by the salivary gland, and its presence can be demonstrated by warming a little 3 per cent. starch solution with a few drops of saliva, and testing with iodine and Fehling solution. The enzyme can be precipitated from saliva in the usual way by means of alcohol. If necessary the secretion of saliva can be stimulated by inhaling a little ether.

The presence of amylase can also be demonstrated in pancreatic extract.

Brown and Morris have exhaustively investigated the conditions of formation of amylase in foliage leaves, and the following description from their paper[1] will serve as a very good example of the methods used in this kind of research, and will usefully illustrate the application of the analytical processes described in Chapter V.

A quantity of leaves of *tropaeolum majus* were dried in a steam oven and ten grams of the dried leaves were treated with boiling water. The solution was cooled to 50° C. and digested with a little amylase for two hours. The mixture was then filtered and the filtrate and washings made up to 144 c.c.

The optical activity in a 10 cm. tube was then found to amount to 1·9 divisions. 100 c.c. of the solution also reduced 0·532 gram CuO, which is equivalent to 0·395 gram maltose.

This amount of maltose in a 10 cm. tube will rotate the polarised ray through 1·54 divisions of the scale. The difference between this value and the observed value, viz., $1·90 - 1·54 = 0·36$, must be due to dextrin, amounting in weight to 0·064 grm.

[1] 'A Contribution to the Chemistry and Physiology of Foliage Leaves.' *Journ. Chem. Soc. Trans.* 1893, p. 629.

The total 144 c.c. of solution or ten grams of leaf have therefore yielded :—

Maltose 0·5688 grm.	=	0·5486 grm.	starch
Dextrin 0·0922 grm.	=	0·0922 ,,	,,
	Total =	0·6408 ,,	,,

Ten grams of leaf therefore contain 0·6408 grm. starch or 6·466 per cent. of their weight.

If an appreciable amount of malt extract has been used, a correction must of course be made for it by determining its optical activity and copper-oxide reducing power in a similar manner.

The determination of the actual amount of amylase present is not possible, but comparative determinations can be made by measuring the amount of saccharification which a given amount of tissue can perform under standard conditions in a given time. The starch, it must be remembered, in order that the ' law of proportionality ' may obtain, must always be in large excess.

Brown and Morris investigated thirty-four species of plants ; they found that all of them contained a measurable amount of amylase, the greatest quantities being obtained from *leguminosae*, especially the common pea. They found that the amount of amylase present varied with the environment, the greatest quantity being found when the plant was kept in darkness ; on exposure to light diminution in the quantity of amylase present took place. It is, of course, well known that starch formation in the leaf cells takes place in presence of light ; it appears, therefore, that the digestion of the starch and consequent formation of amylase takes place in darkness ; thus the starch and amylase are present in inverse proportion.

CHAPTER VII

INVERTASE AND MALTASE

Invertase.—Invertase, or sucrase, is the enzyme which brings about the inversion of cane sugar according to the following equation :—

$$\underset{\text{Cane sugar}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Dextrose}}{C_6H_{12}O_6} + \underset{\text{Lævulose}}{C_6H_{12}O_6}$$

Invertase is most readily prepared from beer yeast. A quantity, say ten grams, is thoroughly washed with water with the aid of a filter pump ; it is then mixed with 100 c.c. of water and about 1 c.c. of chloroform ; the chloroform prevents the growth of the yeast. On warming the mixture for a few hours at about 30° C. and filtering, a solution is obtained which contains the enzyme invertase. This can be proved by adding, say, 5 c.c. of the solution to 50 c.c. of a 10 per cent. solution cane sugar and warming to about 30° C. for an hour. Before testing for the presence of invert sugar, the solution should be boiled to remove the chloroform present, which otherwise would tend to reduce the Fehling solution. The boiled solution should be cooled and made up to its original volume with distilled water, and the invert sugar determined by means of Fehling solution and by the polarimeter in the usual way.

It will be seen that the separation of invertase from the yeast has not necessitated the breaking up of the yeast cell ; simple diffusion has been sufficient to extract it. Invertase

is, therefore, an enzyme capable of passing through the cell wall, and thus belongs to the class known as *extra-cellular* enzymes, in contradistinction to other enzymes found in yeast such as *maltase* and *zymase*, which are only obtained if the cell wall is partially, at any rate, broken down.

Invertase can be separated from solution in the ordinary way by precipitation with alcohol, as was first shown by Berthelot. It has been exhaustively investigated by O'Sullivan and Thompson. The best yield of invertase was obtained from yeast liquor, which results when well pressed sound yeast is allowed to stand for some time. A process of self-digestion then sets in, the yeast being converted into a dark-coloured liquid with a characteristic but not unpleasant smell. An addition of 47 per cent. of alcohol to this liquid gives a good precipitate of invertase. They found that the action of invertase on cane sugar proceeded in accordance with the law which has been found to obtain in purely chemical reactions, in which no condition varies except the diminution of the changing substance; i.e., if the quantities of sugar inverted were plotted as ordinates to a curve, and the corresponding times as abscissæ, a definite time curve resulted.

The speed of the reaction was found to increase with the temperature up to 55°–60° C., but at 75° C. the enzyme is destroyed.

Caustic alkalis were found to be instantly destructive of the enzyme, whereas minute quantities of sulphuric acid were favourable to its action. Any excess of acid above a defined minimum was, however, detrimental in its effect.

There appeared to be no limit to the activity of the enzyme, as a sample of invertase which had inverted 100,000 times its own weight of sugar was still active.

It is noteworthy, in view of the more recent work on maltase, that the products of the reaction appeared to have no effect on its rate; on the other hand, the enzyme can

withstand a temperature 25° higher in presence of sugar than when heated by itself.

The secretion of invertase by a typical mould, e.g., *Aspergillus niger*, can be demonstrated by the following method described by Duclaux.

A quantity of Raulin's solution (see p. 27) should be made up and sterilised by heating several times in the steam steriliser. A large sterile Petri dish about 20 cm. in diameter is taken and filled to a depth of one centimetre with the sterile solution. It is then inoculated by means of a sterile platinum wire with a pure cultivation of *Aspergillus niger* and the whole is allowed to develop for some days; a voluminous growth quickly takes place. When the mould has developed over the surface and has acquired a green or brownish colour, the liquid can be carefully siphoned off from beneath it and the solution replaced by sterile water. On repeating this operation at the end, say, of two days, practically no sugar will be found to be present on testing with Fehling solution. On filtering the solution and warming, say 10 c.c. with 50 c.c. of a 10 per cent. solution of cane sugar, invertion will be found to take place, showing that invertase has been secreted by the organism, and has gone into solution.

Maltase or Glucase.—This enzyme converts maltose into dextrose according to the following equation :—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

Maltase is an enzyme which occurs in yeast, but whose presence is not so easy to demonstrate as that of invertase. The following method is described by Croft Hill (see also Brown's 'Laboratory Studies,' p. 142) : A quantity of ordinary pressed brewer's yeast is well washed by decantation and drained and pressed over the filter pump; it is then finely crumbled in a mortar and further drained from moisture if necessary. About twenty grams are then taken, spread in a thin layer on a porous plate and dried in a vacuum desiccator over sulphuric

acid for two or three days. The dry mass is then powdered very finely in a mortar and transferred to an air-bath, the temperature of which must be raised very slowly (in about two hours) to 50° C., at which point it must be kept for one hour.

To demonstrate the presence of maltase in the prepared yeast, add about 0·5 gram of the powder to 100 c.c. of a solution of about 5 per cent. of maltose of known rotatory power containing 0·5 c.c. of toluene as an antiseptic (chloroform must not be used, as it prevents the action of maltase). Cork the flask containing this solution and keep it at a temperature of 35° C. for three or four hours. The solution is then filtered and examined in the polarimeter. A considerable fall in the rotation will be found to have taken place, due to the formation of dextrose ; the presence of dextrose may be confirmed by preparing its osazone.

The action of maltase upon maltose is of very special interest, as it is the first case of a reversible enzyme action that has been studied. Croft Hill found that if maltase was added to a very concentrated solution of dextrose a disaccharide was formed. He at first thought that this was a simple reconversion of dextrose into maltose, but further research showed that the sugar formed was isomeric with maltose. The essential fact remained that while in dilute solutions there was a breaking down of larger into smaller molecules, in concentrated solutions there was a building up or synthesis of the simpler molecules into more complex. This would seem to indicate that all enzyme actions are potentially reversible, and the direction of the reaction depends on the concentration of the solution and the relative masses of the reacting bodies ; thus in solutions of less than four per cent. of dextrose no formation of disaccharose occurred.

Subsequent to Croft Hill's researches other instances of reversible enzyme action have been discovered. Thus Fischer and Armstrong have found that isolactose can be

synthesised in the presence of the enzyme lactase from a mixture of equal proportions of glucose and galactose ; and certain fat-splitting enzymes have been found to act reversibly, but the difficulty of working with very concentrated solutions limits the number of successful experiments in this direction.

The importance of such synthetic reactions cannot be over-estimated, as we see here a possibility of bringing about reactions by methods closely akin to those by which the synthesis of natural substances is effected by the living organisms, whether plant or animal.

CHAPTER VIII

THE ALCOHOLIC FERMENTATION OF GRAPE SUGAR

It has already been shown how by the action of the enzyme invertase, secreted by the yeast cell, ordinary cane sugar takes up the elements of water to form a molecule of dextrose and a molecule of lævulose according to the equation :—

$$C_{12}H_{22}O_{11} + H_2O = \underset{\text{Dextrose}}{C_6H_{12}O_6} + \underset{\text{Lævulose}}{C_6H_{12}O_6}$$

and it was shown how this enzyme could readily be extracted from the yeast. If yeast is allowed to develop in a solution of sugar an entirely different and more profound change takes place. This may be demonstrated by the following experiment.

About eight grams of cane sugar are added to about 200 c.c. of water in an ordinary half-litre flask, and about 1 c.c. of fresh brewer's yeast added. The flask is then placed in an incubator at a temperature of 24° C., and after some time an effervescence of gas takes place. If a stopper with a bent tube is attached to the flask and the tube led below the surface of a little lime water, the latter will turn milky, showing that the gas evolved consists of carbon dioxide. The contents of the flask after fermentation has continued for some time will be found to have an alcoholic smell. If the flask is now attached to a Liebig's condenser, and placed on a water-bath, the alcohol can be distilled over. It is possible more simply to demonstrate its presence by attaching a long tube to the

flask; on heating the latter on the water-bath alcohol will be seen first of all to condense in the tube, and afterwards to pass off as vapour, which can be easily detected by applying a light, when the characteristic non-luminous flame of alcohol is produced. This is the *alcoholic fermentation* of sugar which is the foundation of the great brewing and distilling industries. As it is of great technical and, one might add, social importance, it has been studied from the very earliest times, and only recently great additions have been made to our knowledge of it. The history of the subject is very largely the history of fermentation, and some brief account of the older theories of this process will not only be of interest in itself, but may enable the full bearing of modern investigations to be better understood.

Alcoholic fermentation has been known from the very earliest times; the preparation of beer from barley, of wine from grapes and the leavening of dough are mentioned in the oldest known writings. By the alchemists alcoholic fermentation was much studied; the Philosopher's Stone was considered to be a kind of ferment. No very clear ideas were, however, possessed by the alchemists in regard to what took place, and a confusion existed in their time between fermentation and effervescence, which were not properly distinguished till the middle of the seventeenth century.

The great medical chemist Libavius (1595) considered that fermentation was a process akin to digestion, a guess the true bearing of which it is hardly likely that its author properly appreciated.

An even more happy suggestion was made in 1648 by Van Helmont, who stated that out of the ferment something passes into the fermenting liquid, which grows in it as a seed.

The authors of the phlogistic theory of combustion, Becher and Stahl, paid attention to alcoholic fermentation. Becher showed that the juice of grapes does not ferment if the skin of the grape is unruptured, and thus showed that alcohol was not

pre-existent in grape juice as had been imagined, e.g., by the alchemist Basil Valentine. Becher considered that air was necessary for the process ; according to the phlogistic theory, an unknown substance, phlogiston, was set free on combustion. As air was necessary, according to his theory, for fermentation, he regarded it as a species of combustion in which likewise phlogiston disappeared. The exact methods of Lavoisier and Cavendish threw light upon this problem, as upon the simpler problems of combustion and all chemical combinations in general. Cavendish determined the amount of carbon dioxide given off from a known weight of sugar. Lavoisier weighed both the alcohol and the carbon dioxide. He thought at first that they exactly made up the weight of the sugar taken, and, his mind filled with the chemistry of oxygen and the formation of oxides by combustion, he regarded sugar also as an oxide splitting off into two simpler oxides, that is alcohol and carbonic acid, by fermentation. He thought at first that the yeast suffered no change, but found that this was not the case, and recognised further that the breaking up of the sugar was not so simple as he had at first imagined, certain by-products in addition to the main substance formed being always present.

Gay Lussac in 1810 contributed some very interesting experiments ; although these did not, in the light of subsequent investigations, confirm the conclusions which he drew from them, yet they are highly instructive. He exposed some grapes with unbroken skins to hydrogen gas so as to eliminate all oxygen from their surface, he then expressed the juice into a vessel over mercury in such a way that no air could gain access. So long as air was not present, no fermentation took place, but immediately oxygen was pumped into the vessel, fermentation arose. He was also able to prevent fermentation of grape juice by confining it in an atmosphere of sulphur dioxide; he naturally concluded that oxygen was an essential factor in the fermentation process, and that in

its absence no change would take place. He considered that oxygen set up, as it were, a movement among the particles of the ferment which was communicated throughout the liquid. The true explanation of Gay Lussac's results was reserved for later investigators.

It was Cagniard de Latour who made a careful examination of the fermentation process and suggested that the decomposition of the sugar was due to the growth of yeast. Shortly after this began the long conflict of opinion between the supporters of the purely biological and of the purely chemical theory of fermentation. It was in 1836–37 that Schwann furnished his famous experiment of passing air through red-hot tubes and afterwards into fermentable solutions, when no change took place. Gay Lussac's notion that fermentation was due to oxygen was thus shown to be untenable. Schwann concluded that fermentation must be due to living organisms suspended in the air, which were destroyed when they passed through a red-hot tube.

The great authority of Liebig was thrown on the side of the purely chemical explanation of fermentation. It was he who developed the idea of *catalysis*, a word already invented by Berzelius. Liebig compared fermentation changes to such catalytic actions as have been mentioned in the first chapter of this book, e.g., the effect of finely divided platinum in bringing about the union of gases at low temperatures, etc. He considered the ferment or catalyst to be itself in a state of unstable equilibrium or decomposition, which it communicated to its surroundings, producing chemical change, as the additional snowflake may precipitate an avalanche. To Liebig's purely chemical explanation were opposed the famous researches of Pasteur and Tyndall on the possibilities of spontaneous generation. Briefly Pasteur's method was to boil fermentable solutions in flasks provided with finely drawn out necks, which after the solution was boiled would either be sealed or bent in such a way that germs could not enter.

Tyndall allowed the open ends of flasks containing boiled fermentable solutions to communicate with a chamber whose walls were coated with glycerine, and the air in which had been allowed to be at rest for some time; in this way all the germs present settled and were fixed by the glycerine. That the space was free from germs was proved by passing a strong beam of light, as explained on p. 8.

The researches of Pasteur and Tyndall corroborated one another: no fermentation took place in Pasteur's boiled flasks when the precaution was taken to prevent subsequent access of germs; similarly no fermentation took place in Tyndall's flasks when the beam of light showed the air above them to be germ free. Pasteur, therefore, contended that no fermentation took place without an organism, and he even went further, and stated that for any given fermentation a specific organism must be present. Liebig remained unconvinced; he found that while no fermentation occurred in a solution seeded with yeast after filtration through a membrane, yet an extract of meat similarly filtered became putrid. Moreover, Liebig quoted his own experiments, in conjunction with Wöhler, on the decomposition of oil of bitter almonds into benzaldehyde and grape sugar by a substance contained in the almond, which we should now call an enzyme. He considered that a substance of a like character must be secreted by the yeast, and that the only connection between the physiological development of the yeast and the phenomena of fermentation is the production in the living cell of a substance which, acting as a ferment or catalyst, effects the decomposition of the sugar.[1]

Liebig died in 1873 before the publication of the recent researches, which have provided an explanation of the apparent contradiction between the purely vital or physiological theories of Pasteur and his own purely chemical point of view.

The development of enzyme chemistry has been to a large

[1] *Ann. Chem. Pharm.*, 153, 1870, p. 6.

extent independent of advances in bacteriology, being more intimately related with physiology, both animal and vegetable; thus the gastric juice of birds was studied by Réaumur and the Abbé Spallanzani in the latter part of the eighteenth century.

In 1822 Dubrunfaut published experiments showing that the saccharification of starch was due to a small quantity of active substance secreted by the barley grain; he, in fact, discovered the existence of what we now term *amylase*. This work was followed up later in 1833 by Payen and Persoz, who discovered the method of precipitation by alcohol now generally used for the preparation of enzymes. Allusion has also been made to the decomposition of the glucoside amygdalin by an enzyme which is known as emulsin. All these, it will be seen, are products of the activity of cells of highly organised animals or plants. The earliest instance of the isolation of an enzyme from a micro-organism is the case of *urease*, or the ferment which converts urea into ammonium carbonate, and which was shown by Musculus to be present in the dead cells of the organism *micrococcus ureae*, which develops in putrid urine.

The isolation of invertase from yeast was dealt with in Chapter VII. It was originally discovered in the early part of the nineteenth century by Döbereiner and Mitscherlich, and isolated later by Berthelot by precipitation with alcohol.

It is only comparatively recently, however, that an enzyme has been discovered capable of producing alcoholic fermentation in solutions of grape sugar. Invertase is capable to a large extent of being washed out of the yeast cell without rupture of the cell wall. In 1897 Buchner of Munich, by employing drastic measures for breaking down the yeast cells and expressing the juice, was enabled to prepare a solution which would cause alcoholic fermentation to take place in solutions of cane sugar.

Buchner's method was as follows: 1000 grams of brewer's yeast were carefully mixed with an equal weight of quartz

sand and 250 grams of infusorial earth generally known as *Kieselguhr*, and the mixture was ground together till plastic and damp; 100 grams of water were added to the mixture, and it was then wrapped up in a press cloth and put in a filter press capable of exerting a pressure of 400 to 500 atmospheres. About 300 c.c. of juice were thus obtained. The remaining cake was ground up again, sieved, and another 100 c.c. of water added; on again pressing a further 150 c.c. of juice were obtained. The whole volume of juice was clarified by shaking with *Kieselguhr* and filtering.

Thus prepared, the juice is a clear opalescent liquid of a pleasant yeast smell, with a specific gravity at 17° C. of 1·0416. On boiling, a quantity of albuminoid matter separates and the liquid becomes nearly solid.

If the unboiled juice is mixed with an equal volume of concentrated cane sugar solution, an evolution of carbon dioxide begins after a period varying from a quarter of an hour to an hour, and the evolution continues for about twenty-four hours.

Similar results are obtained from grape sugar and from fructose, but not from lactose or mannitol; this corresponds with the activity of the living yeast. Every precaution was taken to work aseptically, and no yeast cells could be found on microscopical examination of the liquid. Moreover, the action is not stopped by chloroform, nor by passage of the liquid through a Berkefeld filter or through a dialysing membrane. Hydrocyanic acid stops the action, but this recommences if air is passed through to drive off the HCN, showing that the effect of the latter is not due to the poisoning of the living organism, but more probably to the formation of a loose compound between HCN and the enzyme.

The fermentation is a true alcoholic fermentation in that alcohol and CO_2 are produced in the same proportion as when living yeast is used; by-products such as succinic acid and glycerine are also produced. The enzyme which is present in the solution has been termed by Buchner **zymase**.

The preparation of yeast juice by Buchner's method requires special apparatus for obtaining high pressures which is not to be found in every laboratory. It is possible, however, to demonstrate the power of alcoholic fermentation, which is possessed by yeast apart from its ordinary vital activity, by making use of a preparation described by Albert in 1900, and known as *permanent yeast* ('Dauerhefe') or more recently *zymin*. This is prepared in the following way: Yeast is rubbed into a powder and brought into a mixture of alcohol and ether, filtered over the filter pump, and again submitted to the same process of digestion with alcohol and ether and filtering. It is then washed with alcohol and ether and finally with dry ether; on allowing the ether to evaporate at the ordinary temperature zymin is obtained as a fine impalpable powder. On examination under a high-power microscope it will be found that the finer structure of the yeast cell has disappeared.

If, now, a small quantity of this powder is ground up with a few c.c. of a warm solution of sugar and a little sand, and the mass poured into a narrow tube, say about 5 mm. wide and 20 cm. long, the whole being then placed in the incubator and kept at a temperature of about 27° C., an evolution of gas will be observed in about half an hour, and with larger quantities the presence of alcohol can be detected in the usual way.

This preparation of zymin is termed permanent yeast, because, in contradistinction to the yeast juice of Buchner, it will retain its activity for a prolonged period. Yeast juice, on the other hand, rapidly loses its activity on standing, and such inactive yeast juice is further characterised by the fact that it gives no precipitate on boiling, that is to say, that the albumin content of the juice has been broken down. It would appear, therefore, that in addition to zymase the yeast juice contains another enzyme which is capable of digesting albumin, that is, a proteolytic, or, to use Armstrong's nomenclature, proteoclastic enzyme; this enzyme would seem to digest not only the albumin present but also the zymase.

These facts have led to the very interesting series of researches by Harden and Young. Harden showed that if an equal volume of blood serum was added to the yeast juice, digestion of the yeast albumin did not proceed so rapidly and the activity of the zymase was increased, that is, there was a more prolonged alcoholic fermentation. Harden and Young further found that, besides serum, boiled yeast juice greatly increased the alcoholic fermentation; thus the total fermentation produced by 25 c.c. of yeast juice acting on 2·5 grams of glucose, was on the average doubled by the addition of an equal volume of boiled juice, and increased to a maximum when three to five volumes were added, after which it decreased.

It might be contended with equal justice either that this increase of fermentation was due to an increase in the activity of the zymase, to decrease in the activity of the proteolytic enzyme, or to a combination of these causes. As a matter of fact, the true cause is being found to lie rather deeper than might at first sight be concluded. When fresh yeast juice is boiled there is, as has been stated, a heavy precipitation of albuminous matter; if this is filtered off, the filtered juice still increases the activity of the zymase; this would seem to indicate that the increased activity was not an enzyme effect, as enzymes in general are destroyed by boiling. The unknown substance is besides capable of passing through a dialyser, but, on the other hand, is precipitated by 75 per cent. alcohol.

It was possible by an ingenious experiment to show that the alcoholic fermentation certainly depends on two substances, neither of which is capable alone of causing fermentation. By soaking an ordinary Chamberland filter candle (such as is often attached to household water taps for the purpose of removing organisms from the water before drinking) in melted gelatine and allowing the latter to set in the pores of the filter, it is possible to obtain a fairly rapid dialysis of colloidal matter by filtering a solution containing such matter through this gelatine filter, under high pressure. This method of

dialysis under pressure was suggested by Dr. Martin, the director of the Lister Institute, and the filter is known generally as a Martin filter. If, now, yeast juice is passed through such a filter a residue is obtained soluble in water, and it is found that neither this residue nor the liquid which passes through the filter are either of them separately capable of causing alcoholic fermentation. On the other hand, when brought together the mixture produces fermentation almost equal to that in the original juice.

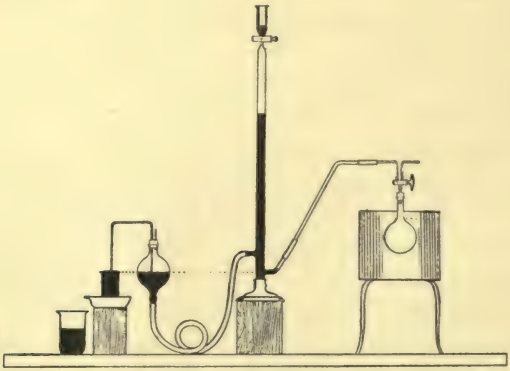


FIG. 22.—APPARATUS FOR MEASURING RATE OF EVOLUTION OF CO_2.

The proteolytic enzyme, as might be expected, remains behind on the filter with the rest of the colloidal matter, and on adding the residue to water, digestion of the albumin rapidly proceeds. The addition of the filtered juice does not increase this effect. So far, then, it is clear that the alcoholic fermentation is due to at least two substances, one of a colloidal and the other of a crystalloidal nature.

It should be explained that in studying the amount and rate of alcoholic fermentation, the evolution of CO_2 is taken as a measure of this change. This rate of evolution is measured in the special apparatus (Fig. 22).

The fermenting mixture is contained in the round-bottomed flask, placed in a constant temperature water-bath. The CO_2 is collected over mercury in the graduated burette, a constant pressure being maintained by the simple compensating arrangement shown in the diagram.

Table III will illustrate the results already described :—

TABLE III

(i) The effect of the addition of boiled juice

—	CO_2 evolved in presence of	
Volume added	Water	Boiled Juice
	gram	gram
1	0·19	0·33
4	0·17	0·53
6	0·14	0·65

(ii) Experiment on the filtration of yeast juice :—

—	CO_2 evolved in presence of			
No. of Experiment	Original juice	Residue	Filtrate	Mixture of Residue and Filtrate
	gram	gram	gram	gram
1	—	0·013	0	0·068
2	0·0704	0	0·001	0·051
3	0·0704	0·001	0·008	0·064
4	—	0	0·007	0·040

The question now remains, what is the nature of the dialysable matter, or co-ferment, as it has been termed, which is necessary for the activity of the enzyme, which

latter presumably is contained in the nondialysable residue and is capable of digestion by the proteolytic enzyme also present therein. Harden and Young's further researches have shown that phosphoric acid is at any rate a necessary constituent of this dialysable substance. On addition of a phosphate to unfiltered yeast juice a great increase of fermentation is obtained. On the other hand, this effect is not produced if the phosphate is added to the residue or to the filtrate separately, and consequently the phosphate, though apparently necessary to the reaction, is not the initially active agent. Moreover, the phosphate does not affect living yeast. It appears that the phosphate actually takes part in the fermentation reaction, and that for every molecule of sugar which is broken down into alcohol and carbon dioxide, a molecule of a complex hexose phosphate, a compound of a sugar molecule with two of phosphate, is simultaneously formed. This compound has actually been isolated. The ordinary fermentation involves the phosphate present in ordinary yeast juice, either as hexose phosphate or free phosphate, and this phosphate passes repeatedly through the cycle of changes represented in the following equations :—

(1) $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$

(2) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$

The hexose phosphate as is shown in equation (2) is hydrolysed with the production of free phosphate, which again undergoes reaction (1), partly with the sugar formed at the same time, and partly with fresh sugar from the solution. The rate at which the second of these reactions occurs determines the rate of fermentation observed when glucose is fermented by yeast juice, which is therefore a measure of the rate at which phosphate is being formed in the juice.

The fermentation of mannose and fructose in the presence of yeast juice has also been examined by Harden and Young. They discovered that while mannose behaves towards yeast juice in the same manner as glucose both in presence and absence of added phosphates, fructose is much more rapidly fermented in the presence of phosphates than either of the other two sugars.

An excess of phosphates lowers the rate of fermentation of glucose and mannose by yeast juice, but an addition of fructose to the fermenting mixture under these conditions has the effect of inducing a rapid fermentation of the other sugars. Fructose in this case appears to act as a catalyst. The addition of glucose or mannose under similar circumstances has no similar effect.

The precise part played by the fructose in this interesting change is not yet fully elucidated.

The fermentation produced by yeast juice is, of course, not exactly the same thing as the fermentation which results from the activity of the living yeast cell. The respiratory, as distinct from the fermentative, activity of the yeast has also to be considered. While Harden and Young's researches are of the highest value as an intimate study of a detached portion of the problem, the study of the conditions of activity of the living cell is, of course, also necessary for a complete solution of the question.

The labours of earlier workers in this field have been supplemented in recent years by the researches of Slator.

He determined the rate of fermentation by measuring the change of pressure due to evolution of carbon dioxide.

He found that in comparatively small intervals of time the rate of fermentation was proportional to the amount of yeast taken, and was independent of the concentration of the sugar except in very dilute solutions.

The interesting observation was made that while galactose is not fermented by yeasts grown in other solutions, it is

possible to acclimatise certain yeasts by growing them in a mixture of this sugar and dextrose, after which they will attack galactose readily.

The results of the experiments on the fermentation of different sugars by yeasts lead to the conclusion that the enzyme of the yeast combines with the sugar, and that the velocity of formation of carbon dioxide is determined by the rate of decomposition of the compound formed.

It is still somewhat an open question whether there are present in yeast cells a large number of enzymes, each capable of exerting its own specific action, or whether only a few enzymes are present, and that the same enzyme can promote different chemical actions.

From the foregoing pages it is evident that the chemistry of the yeast cell has been a fruitful subject of inquiry. The researches that have been considered are of great scientific interest in showing the complexity of the reactions which take place even under the comparatively simple conditions afforded by a single cell of yeast.

They have also a very important bearing on the fermentation industries, which have for their object the preparation of various forms of alcoholic beverages.

While it is impossible usefully to consider these in this book, owing to the complexity of their purely technical detail, mention should be made of the great advance made in the brewing industry by the use of pure cultures of yeasts introduced by Hansen.

His method of obtaining these on a small scale has been described in Chapter II. By successive inoculation into larger and larger volumes of sterile wort it has been possible to brew beer by means of one culture only. The brewer is enabled thus to conduct the process of fermentation under rigidly controlled conditions.

CHAPTER IX

THE ACID FERMENTATION OF ALCOHOLS AND CARBOHYDRATES

It is probable that the earliest fermentation known to man was the souring of milk; this we now know to be due to the fermentation of milk sugar, and it is one of the more important of a class of fermentation changes, all of which essentially consist in the oxidation of the characteristic alcohol group CH_2OH to the group characteristic of acids, viz. CO_2H or carboxyl, either by addition of oxygen or by intra-molecular change.

The simpler carbohydrates or sugars are, as we have learnt, ketone or aldehyde alcohols, and therefore lend themselves to this change.

The oxidation of the alcohol group can of course be brought about by purely chemical reactions. The chemical method which is of most interest in the present connection is the oxidation of alcohols by means of platinum black; the latter is obtained as a black precipitate when solutions of platinum salts are treated with certain reducing agents. This finely divided platinum, to which reference has already been made on p. 3, has the power of causing oxidisable vapours to combine with oxygen when the two are led over it together; thus, e.g., ordinary formalin or formaldehyde is prepared by bubbling air through methyl alcohol, and leading the mixture of air and methyl alcohol vapour over platinum black. In this case indeed it is sufficient to heat a spiral of platinum wire, and plunge it into the mixture of methyl alcohol vapour and

air, for the reaction to begin. The wire continues to glow so long as the gases pass over it.

The oxidation of alcohol vapour by means of platinum black can be shown by the following simple experiment.

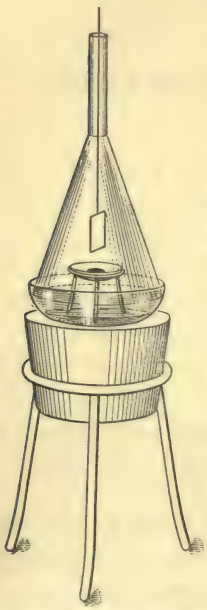
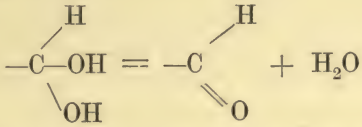


FIG. 23.—THE OXIDATION OF ALCOHOL VAPOUR.

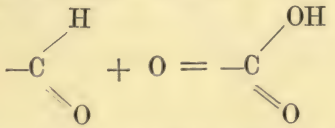
A wide shallow porcelain dish is placed upon a water-bath and a little alcohol poured in, about a gram of platinum black is placed in a watch glass resting on a small tripod, the whole is covered by a large inverted funnel, through the neck of which a piece of blue litmus paper is suspended (Fig. 23). On gently warming the alcohol it vapourises and oxidation takes place at the surface of the platinum black; aldehyde, and finally acetic acid, being obtained, the presence of which is rendered evident by the reddening of the litmus paper. Care must be taken to vapourise the alcohol very slowly, or oxidation may take place with explosive violence.

The oxidation of alcohols by means of platinum black has been dwelt on at some length because it offers the nearest analogy to bacteriological or enzyme reactions. There are good reasons for thinking that the progressive oxidation of an alcohol to an acid takes place by addition of oxygen, through the formation of additional hydroxyl groups, and subsequent elimination of water. Thus the addition of oxygen to the group $-CH_2OH$ may be considered to result first in the formation of the group $-C(H)(OH)(OH)$; such a combination is

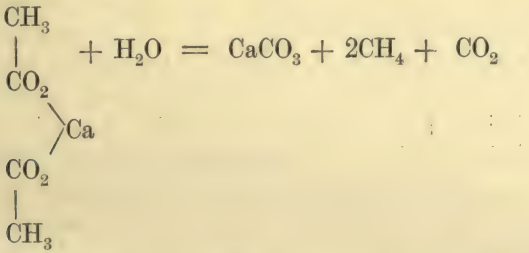
unstable, and water is eliminated with formation of the aldehyde group.



Further addition of oxygen gives rise to an acid, thus :—



If the oxidation is carried still further, hydrocarbons and carbon dioxide (CO_2) generally result. E.g., calcium acetate undergoes fermentation with formation of calcium carbonate and marsh gas, thus :—



Up to the present no specific enzymes have been discovered capable of bringing about separate and defined reactions of any of these types ; few, if any, of the reactions are confined to specific bacteria, consequently the oxidation of an alcohol through the intervention of the living organism is a highly complex process, generally resulting in the production of a number of secondary products. The action of an organism, as has been frequently stated, may be broadly described as respiratory and fermentative. It consumes a certain amount of the medium for building up its own structure ; in such a

case ultimate products, such as CO_2 and other gases, result. Incidentally, as it were, more of the medium has to be broken up than actually suffices for the food of the organism, and we thus get the normal products of fermentation. The course of reaction, therefore, in every case depends on several factors, viz.:

1. The nature and molecular constitution of the fermentable substance, whether an alcohol, aldehyde or ketone, etc.

2. Whether any other food supply is present, thus, e.g., the character of the decomposition of a sugar has been found to vary according to the presence or otherwise of peptone in the nutrient mixture.

3. The species and state of growth of the organism; for instance, results will vary according as the culture is or is not of recent growth, or according to whether it comes from strains which have been transplanted from time to time in the laboratory.

A complete account of all the oxidation changes of the type under consideration, and of the bacteria concerned therein, would lead too far and would be of doubtful utility, inasmuch as many of them have not been worked out in detail. Reference will be made in the first place to three fermentations of technical importance, and afterwards some account will be given of the detailed work in the case of specific organisms which will serve to illustrate the method of research used in this class of inquiry.

The Oxidation of Alcohol to Acetic Acid.—The simple equation expressing this reaction is as follows :—

$$CH_3CH_2OH + O_2 = CH_3CO_2H + H_2O$$

In reality, for reasons mentioned above, the bacterial oxidation of alcohol is by no means capable of so simple an expression.

It is well known, of course, that alcoholic liquids such as wine and beer, on exposure to air, gradually become sour.

The true explanation of this phenomenon was afforded by the researches of Pasteur, though others, e.g., Person in 1822, had noticed the growth of organisms as a fine film on the surface of such a liquid and had given the name *Mycoderma aceti* to the growth.

Pasteur showed that certain rod-like bacteria were the true causes of the formation of acetic acid, while other organisms which might be present, such as yeasts, etc., carried the oxidation further to CO_2 and H_2O. Hansen was the first to obtain pure cultivations of *Mycoderma aceti*, and discovered also further species capable of bringing about the same change. As a matter of fact, as already indicated, quite a large number of organisms can effect the formation of acetic acid, not only from ethyl alcohol, but from other alcohols and carbohydrates which contain the characteristic group CH_2CH_2OH.

It should be pointed out that the formation of acetic acid by bacterial action can only take place within certain limits of concentration, and in presence of the essential ingredients of bacterial food, that is, nitrogen must be present in some form, e.g., as peptone or albumin, and phosphorus as phosphate.

The Lactic Acid Fermentation.—As already mentioned, the souring of milk is due to the formation of lactic acid by decomposition of milk sugar. The simple chemical equation in this case is as follows: The milk sugar is first inverted, forming two hexose molecules—

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

By a simple molecular decomposition one hexose molecule yields two molecules of lactic acid, thus :—

$$C_6H_{12}O_6 = 2C_3H_6O_3$$

The production of lactic acid from milk can be brought about by the addition of a small quantity of previously soured milk. The reaction quickly reaches a limit if the solution is allowed to become too acid, and therefore chalk is generally

added to neutralise the free acid as it is formed, calcium lactate being the result.

Under certain conditions a further decomposition of the lactic acid occurs, forming *butyric acid* according to the following equation :—

$$\underset{\text{Lactic acid}}{2C_3H_6O_3} = \underset{\text{Butyric acid}}{C_4H_8O_2} + \underset{\text{Carbonic acid gas}}{2CO_2} + \underset{\text{Free hydrogen}}{2H_2}$$

This **butyric fermentation** is brought about by a number of organisms, some of which are anaerobic.

The equation given above, representing the formation of lactic acid from a hexose, must only be taken as a part of what actually occurs. Moreover, in the case of lactic acid, there are in this simple equation further possibilities because, as already explained, lactic acid contains an asymmetric carbon atom, and therefore exists in three possible forms, viz., a right-handed and left-handed, and an inactive modification. Which of these forms remains at the end of the reaction depends on the conditions of experiment. It will be remembered that the lactic acid above referred to is the *a*-acid $CH_3\,\mathbf{C}\,HOHCO_2H$, the central carbon atom being asymmetric ; this is the form almost invariably met with as the result of lactic acid fermentation. There is also, it may be remembered, another form of lactic acid, viz. *β*-lactic acid, $CH_2OHCH_2CO_2H$, the production of which is a further possibility. Its production has been stated to occur when inosite is fermented under certain conditions, but the evidence of its occurrence is somewhat conflicting.

The chemical interest of the lactic acid fermentation centres, therefore, generally round the conditions of production of the right-handed and left-handed modifications, and reference may therefore be made to the experiments of Frankland and Macgregor, which indicate that the inactive or racemic form of acid may in certain cases be produced, after which a preferential decomposition of one of the modifications takes place.

Frankland and Macgregor experimented with a bacterial

growth which had the power of exciting a vigorous fermentation in suitable solutions of calcium lactate. The composition of the medium was as follows :—

Calcium lactate,	3 grams	made up to 300 c.c. with distilled water.
Peptone solution,	30 c.c.	
Calcium carbonate,	3 grams	

This solution was inoculated with a minute quantity of calcium lactate solution in active fermentation.

The quantity of nutrient solution, its concentration (3 per cent. instead of 1 per cent., as above, being occasionally used), and the duration of the fermentation were varied in different cases. At the end of each experiment the calcium carbonate was filtered off and the filtrate concentrated and examined in the polarimeter. The calcium was removed from solution by means of oxalic acid, and the filtrate from the calcium oxalate evaporated on a water-bath to remove volatile acids. The lactic acid remaining was separated from other impurities by precipitation with lead acetate, decomposition of the lead salt with H_2S, evaporation of the filtrate from the lead sulphide, and extraction with ether. The residue after evaporation of the ether was converted into the zinc salt by boiling with zinc carbonate, and the solution of the zinc salt was again examined in the polarimeter. This zinc salt was found to be pure lævo-rotatory zinc lactate. The calcium lactate originally taken was inactive, so that there had evidently been preferential decomposition of the dextro salt. If the fermentation was stopped at too early a stage, the active lactate was found to be mixed with a large quantity of inactive lactate, whilst when the fermentation was too long continued, the active lactate was also destroyed. The above description will serve to illustrate the kind of investigation necessary for determining the precise products of a reaction, when there is a possibility of one or another stereo-chemical modification being produced. The production of an inactive or active modification

depended in this case on the organism. With different conditions of experiment different results are obtained, and the production of a dextro or lævo form depends quite as much on the fermenting medium as on the organism producing the fermentation. The dependence of the products of the reaction upon the constitution of the fermenting molecule has been the subject of a very interesting research by Harden, who has

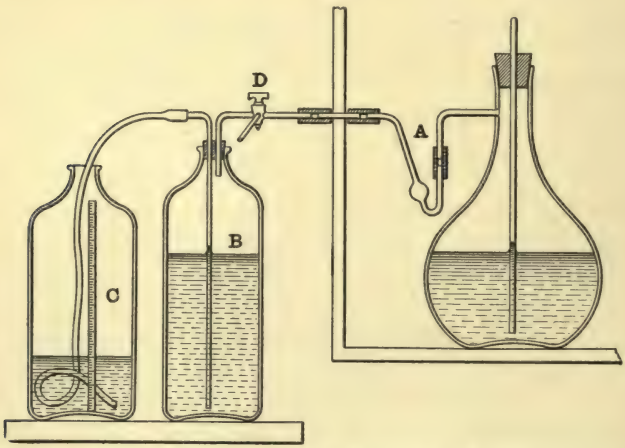


FIG. 24.—APPARATUS USED IN DR. HARDEN'S EXPERIMENTS ON *B. coli communis*.

studied the chemical action of *B. coli communis* and similar organisms on carbohydrates and allied compounds.

The apparatus shown in Fig. 24 was made use of; one litre of the solution was placed in a large flask provided with a side tube and an indiarubber stopper, through which passed a straight glass tube leading to the bottom of the flask, the side tube and vertical tube were plugged with cotton wool and the flask then sterilised. The side tube was then attached to a piece of bent tubing (A) on which a small bulb was blown near the bend, a drop of mercury was placed in the tube and served

to seal the apparatus and prevent diffusion, whilst at the same time it readily allowed gases to pass out of the flask. After inoculation, which was effected by removing the stopper and introducing a loop full of the culture, the air of the flask was displaced by nitrogen, prepared by the action of ammonia solution on copper.

After passing nitrogen for about one to two hours, the flask was removed and the long vertical tube sealed off at a constriction previously made near the top. It was then placed in an incubator (Fig. 24), the side of which was pierced by a brass tube, with which the tube A is connected by indiarubber tubing. The apparatus for collecting and measuring the gas was connected with the other end of the brass tube. It consisted of a Winchester quart bottle (B), fitted as an aspirating bottle, and provided with a long piece of indiarubber tubing passing to the bottom of a second bottle, graduated in volumes of 100 c.c. on a piece of paper pasted to the glass. On the tube between the flask and the collecting bottle was placed a three-way tap (D), by means of which samples of gas can be withdrawn either directly from the flask or from the collecting bottle. The collecting bottle was filled with saturated brine, on the surface of which a little oil was poured, to prevent absorption of carbonic acid gas. Direct experiment showed that a mixture of carbonic acid gas and air could be preserved over this liquid for a considerable time without undergoing any perceptible alteration in composition.

About 100 c.c. of brine were placed in C, and the connecting rubber tube was also filled with brine so that the volume of gas evolved could be measured by that of the liquid displaced.

During the period of incubation the flask was agitated at frequent intervals in order to secure the neutralisation of the acid produced, and the volume of the liquid displaced was read off, the measuring bottle being raised or lowered until the surface of the liquid in it was at the same level as that in the collecting bottle.

As soon as about two litres of gas had been collected a sample of about 500 c.c. was taken for analysis. The remainder of the gas was swept out through the three-way tap by raising the measuring bottle, and the apparatus then arranged as before for the collection of a fresh quantity of gas.

At the close of about fourteen days the flask was removed from the incubator, and a culture made on agar, which was examined and in every case found to give the usual tests for normal *B. coli communis,* or other organism studied. The solution was then measured, and aliquot portions removed for the estimation of the various constituents.

These products comprised :—

Lactic acid,
Succinic acid,
Acetic acid,
Ethyl alcohol,
Formic acid,
Carbon dioxide,
Hydrogen.

The effect of various nitrogenous products serving as sources of nitrogen for the organism was also studied. It was found that Witte's peptone was the best source of nitrogen to employ, as the products of its decomposition are not sufficient to interfere with the estimation of those produced from the special compound under examination.

The general method of preparation of the medium under examination was as follows: 10 grams of Witte's peptone were boiled with tap water, 20 grams of the sugar or other compound to be examined were added, together with 10 grams of pure calcium carbonate, the whole being made up to one litre ; in some cases 2 grams of calcium phosphate were added, but no beneficial effect could be observed. Harden found that glucose yielded chiefly lævo-lactic acid together with 5·25 per cent. of the inactive form ; fructose, arabinose and galactose behave similarly. On the other hand, mannite

yields a greater percentage of laevo acid and is especially distinguished by the fact that nearly 25 per cent. of the weight of mannite fermented appears as ethyl alcohol, or more than twice as much as in the case of the sugars studied.

The general equation for the decomposition of glucose which may be considered as typical is as follows :—

$$2C_6H_{12}O_6 + H_2O = 2C_3H_6O_3 + C_2H_4O_2 + C_2H_6O + 2CO_2 + 2H_2$$

Harden concludes that the products of the reaction can be referred to the constitution of the fermenting substances. Thus the yield of alcohol depends essentially on the presence of the group, $CH_2OHCHOH$; this only occurs once in glucose, whose formula it will be remembered is

$$CH_2OH(CHOH)_4—CHO$$

On the other hand, mannite contains the alcohol-producing group twice, thus :—

$$CH_2OH—(CHOH)_4—CH_2OH$$

and consequently is capable of yielding a greater proportion of alcohol.

The mechanism of the fermentation of glucose as effected by *B. coli* is shown, according to Harden, by the following scheme :—

$$\begin{array}{l} CH_2OH \\ | \\ CHOH\ CH_2OH \end{array} = CH_3—CH_2OH + CO_2 + H_2$$

$$\begin{array}{l} CHOH\ CHOH \\ |\quad\quad| \\ CHOH\ CHOH \\ |\quad\quad| \\ CHOH\ CHOH \end{array} = \text{lactic acid, succinic acid, etc.}$$

$$\begin{array}{l} COH\quad CHOH \\ \quad\quad\ | \\ \quad\quad COH \end{array}$$

The reaction in the case of mannite would be represented as follows :—

$$\begin{array}{l} CH_2OH \\ | \\ CHOH\ CH_2OH \end{array} = CH_3{-}CH_2OH + CO_2 + H_2$$

$$\begin{array}{l} CHOH\ CHOH \\ |\quad\quad | \\ CHOH\ CHOH \\ |\quad\quad | \\ CHOH\ CHOH \end{array}$$

$$\begin{array}{l} CH_2OH\ CHOH \\ \quad\quad\quad | \\ \quad\quad CH_2OH \end{array} = CH_3{-}CH_2OH + CO_2 + H_2$$

The precise modification of lactic acid which may be produced will, according to this theory, depend on the configuration of the three centre CHOH groups and also on the particular organism taking part in the reaction.

Harden's suggestion as to the dependence of the alcohol formation on the presence of a terminal group $CH_2OHCHOH$ finds further confirmation in his studies in conjunction with Walpole on the action of *B. lactis aerogenes* on glucose and mannite. They found that, in addition to the usual products of this class of fermentation, glucose yields butylene glycol, $CH_3CH(OH)CH(OH)CH_3$. Mannite, on the other hand, gives similar products, but less butylene glycol and more alcohol.

The instances given in the foregoing chapter will sufficiently indicate the complexity of the problem involved in obtaining a full explanation of the manner in which carbohydrates break down under the action of bacteria. Comparatively few cases have been worked out in a rigorous manner as in the researches referred to above ; it is, however, only by systematic quantitative work of this kind, with sub-

stances whose chemical constitution can be determined, that a sure advance in our knowledge of the chemistry of vital action is likely to be attained.

The *technical applications* of the activity of acid-forming bacteria are numerous and important.

The production of **vinegar** is due to the activity of various species of bacteria which bring about the oxidation of alcohol to acetic acid. Different qualities of vinegar are obtained according to the process used. In France wine is allowed to become sour in vats which are first filled with vinegar, wine being gradually added, with simultaneous withdrawal of a portion of the vinegar. The wine becomes charged with acetic acid bacteria and is rapidly converted into vinegar, when the withdrawal of the vinegar formed and the addition of more wine is repeated.

A more rapid process is in use in Germany and also in England, according to which dilute alcohol is slowly passed over beech wood shavings contained in large vats, suitably ventilated to allow free passage of air. The shavings are previously sown with acetic acid bacteria. A rapid oxidation of the alcohol takes place.

In the **tannery** acid-forming bacteria also play their part. In order to remove hair from hides, they are generally first soaked in lime, which has to be thoroughly removed from the skin before the tanning process. This removal takes place partly in what is known as the 'puering' or 'bating' process and partly in the subsequent 'drenching.' In the 'puering' process the skins are placed in a bath of dog's dung or similar material, when, in addition to many other changes, e.g., the action of proteolytic enzymes on the albumin constituents of the skin, ammonium salts of butyric and other acids are formed, which exercise a solvent action on the lime. This is completed in the 'drenching' process, where the skins from the bate, after washing, are placed in an infusion of bran. A mixture of organic acids, chiefly lactic,

is produced from the fermenting bran, which removes the last traces of lime.

The very important application of the lactic fermentation to dairy practice will be referred to in the special chapter on the applications of bacteriological chemistry to agriculture.

CHAPTER X

THE FERMENTATION OF CELLULOSE AND ALLIED BODIES

Cellulose, broadly speaking, constitutes the framework of the vegetable world, and when the vast quantity of vegetable matter on the face of the globe is considered, the importance of the changes which accompany its decomposition and absorption into the cycle of life is seen to be of the first importance. Before considering these changes and the conditions of their operation, some brief description must be given of cellulose and its allied substances.

Cellulose can be obtained as a residue after dissolving out the other constituents of plants, by the following experiment:—

Dissolve 30 grams of powdered chlorate of potash in 520 c.c. of cold nitric acid (s.g. 1·1). Suspend in this mixture a number of leaves, stems, etc., and allow them to remain undisturbed at a temperature not above 20° C. until they are perfectly whitened. This may require from two to three weeks.

Pure Swedish filter paper (acido hydrochlorico et fluorico extracto) is practically pure cellulose.

We are indebted for our knowledge of the chemistry of cellulose in large measure to the long-continued and careful researches of Cross and Bevan, from whose works the following information is largely derived.

From its empirical composition cellulose is found to belong to the carbohydrates and its empirical formula is $(C_6H_{10}O_5)_n$.

The composition of the actual cell wall of plants varies greatly, as there is a large variety of substances known generically as cellulose, and having the same empirical composition, but which yet exhibit considerable differences in their physical properties and in their behaviour towards reagents.

Cross and Bevan divided celluloses into three classes according to their behaviour with reagents. The main reagents used in cellulose investigation are strong acids and alkalies, which bring about conversion into sugar by the ordinary hydrolytic change, that is :—

$$C_6H_{10}O_5 + H_2O = C_6H_{12}O_6$$

Acetic anhydride combines with any OH groups which may be present according to a general equation :—

$$2R{-}OH + \left.\begin{matrix} C_2H_3O \\ C_2H_3O \end{matrix}\right\} O = 2R{-}OC_2H_3O + H_2O$$

According, therefore, to their behaviour with these and other reagents cellulose bodies are classified as follows :—

1. Those which offer a maximum resistance to hydrolytic action and which contain in their molecule no directly active CO groups, i.e., the CO is not easily oxidised and does not combine, e.g., with phenyl hydrazine. These are represented by the cellulose of cotton fibre.

2. Those of less resistance to hydrolysis which contain active CO groups, i.e., which will give osazones with phenyl-hydrazine. These are perhaps best regarded as oxycelluloses. They appear to constitute the main mass of the tissue of flowering plants and they exist in conjunction with a substance called lignine in the walls of wheat cells.

3. Those that hydrolyse with some facility, being more or less soluble in alkalies and easily decomposed by acid, with formation of carbohydrates of low molecular weight. Included among these is the cellulose of the walls of the cells of seeds. It will be remembered that in the preparation of

soluble starch, the starch cellulose enveloping the starch granules was destroyed by heating with dilute hydrochloric acid.

Allied with cellulose are kindred bodies belonging to the *pectin* group.

Pectose is the name given to the parent substance of bodies such as pectin, pectic acid, etc.

Pectin can be obtained by filtering the juice of a ripe apple or pear through muslin, and adding an equal bulk of alcohol. The pectin is precipitated as a stringy gelatinous mass, which can be reduced to a white powder soluble in water.

A solution of pectin gelatinises on standing, probably by the action of the enzyme pectase contained in the fruit juice.

The members of the pectose group have chiefly been investigated by the French chemist Mangin, who divides these bodies into two series :—

(1) Neutral bodies which vary in their solubility in water. At one extreme we have the substance pectose, which is insoluble in water and closely associated with cellulose ; at the other extreme the substance known as pectin, which is soluble in water but tends to form a jelly fairly readily. Intermediate between these are bodies of a gelatinous nature.

(2) Substances allied to this group are feeble acids, the chief member being pectic acid, which occurs as calcium pectate ; the latter forms a binding substance between the fibre of many plants.

Pectose bodies differ from cellulose derivatives in being insoluble in Schweitzer's reagent. This is obtained as follows :—

A saturated solution in water is made of equal parts of copper sulphate and ammonium chloride. Strong caustic soda is added till no further precipitate is formed. This precipitate of hydrated copper oxide is dissolved in strong ammonia solution as required.

The solubility of cellulose in this reagent can be tested by warming a few strips of filter paper in half a test-tube full of Schweitzer's reagent until solution is practically complete; on acidification cellulose will be precipitated as a flocculent precipitate. One of the technical processes for the production of artificial silk is based on the solution of cellulose in copper-ammonium solutions, and its re-precipitation under conditions resulting in the production of fine fibres.

It is possible to distinguish microscopically in a plant section between cellulose and pectose, by dissolving out the cellulose with a few drops of Schweitzer's reagent.

The cellulose can be further distinguished from the pectose by treatment with dilute iodine; partially hydrated cellulose, such as can be obtained by treatment of ordinary cellulose with alkali, is stained blue by iodine. E.g., the cellulose precipitated from solution in the foregoing experiment can be coloured thus; pectose bodies give no coloration.

Coming now to the method by which cellulose and pectose bodies are broken down in nature, we find in the case of cellulose that this occurs by three well-defined processes:—

1. By the action of the enzyme *cytase* which is secreted by cells and by various organisms.

2. By fermentation under *anaerobic* conditions, that is, in absence of air, through the action of certain specific bacteria.

3. By decomposition under *aerobic* conditions, through the action of certain bacteria and moulds in presence generally of nitrates.

It is probable, of course, that the action of the organism in the last two cases is due to secretion of a cellulose-dissolving enzyme, but this has not so far been actually isolated.

1. We may take these cases in order. It will be remembered that in the process of saccharification of starch, which takes place in the development of the barley grain, the cell walls of the endosperm are broken down and the interior

of the grain of malt rendered quite friable, and that for this reason it is almost impossible to obtain a section of such a grain. This is due to a cellulose-dissolving enzyme known as *cytase* being secreted by the growing embryo, and the action of this secretion must precede the action of amylase, if the latter is to obtain access to the starch grains confined within the cells of the endosperm. This can be demonstrated by careful microscopic observation of the germinating barley grain, but more simply by the following experiment, which depends on the fact that cytase is destroyed at a temperature above 60° C.

A solution of malt extract is taken and divided into two portions, say of 50 c.c. each. One of these is heated for half an hour at 70° C. In each of the solutions a thin slice of potato is suspended by means of a thin copper wire attached to a glass rod or match stalk placed across the top of the small beaker used for the experiment. A little thymol is added to each of the solutions to prevent the development of moulds or bacteria, and the two beakers placed in an incubator at 40° C. for some days. It will soon be noticed that the slice of potato in the solution in which the cytase has not been destroyed becomes soft and pulpy, the other slice remains quite hard, and the cell walls can be seen by microscopical observation to be quite unattacked.

A similar result can be obtained if an infusion of raw oats is used, and careful investigation by Horace T. Brown has shown that the power of grain-feeding animals to digest such food depends on the enzyme contained in the food, and not on any cellulose-dissolving power possessed by the secretion of the stomach of the animal.

2. The decomposition of cellulose by bacteria in *absence* of air can be demonstrated as follows :—

Some strips of filter paper are placed in a small flask and a few c.c. of deposit from an ordinary sewage septic tank, or

of mud from the bottom of a stagnant pond in which fermentation has been shown to take place by the production of gas on stirring the deposit on the bottom, are added. The flask is filled up with water and attached to a Hempel gas burette. On keeping the flask for some days at a temperature of about 35° C. gas will be evolved, and the filter paper will show signs of pitting, and after the expiration of possibly some weeks will finally be completely disintegrated. On testing the gas it will be found to be inflammable, burning with a non-luminous bluish flame, and if analysed can be shown to consist mainly of marsh gas, CH_4, together with smaller quantities of hydrogen, H, and carbon dioxide, CO_2.

This fermentation has been very carefully worked out by the Russian chemist, Omelianski. For the purpose of his investigation he used Neva mud and pure Swedish filter paper; he was able to isolate two different organisms, one of which produced marsh gas and the other hydrogen. His method of separation depended on the fact that both organisms formed spores, and the spores of the hydrogen organism were able to withstand a higher temperature than those of the marsh-gas organism. On starting the fermentation the marsh-gas fermentation is predominant; by heating the mixture for fifteen minutes to 75° C. at this stage, the marsh-gas organism was killed, but the spores of the hydrogen organism were unaffected. On re-inoculating a fresh quantity of filter paper from the heated solution, the hydrogen organism mainly developed, and by a succession of similar operations he succeeded in obtaining pure cultivations of the two bacteria. They were found to be almost identical in appearance and both produced spores. They differed only in their optimum temperature of reaction and in their resulting products.

He was able to show that the products obtained completely accounted for the weight of paper originally taken, certain fatty acids being produced together with the gas. Thus in the

case of the hydrogen bacillus the following products were obtained from the original weight of 3·3471 grams of paper :—

Fatty acid	2·2402
CO_2	·9722
H	·0138
	3·2262 grams

The marsh-gas fermentation yielded the following products from 2·0065 grams of paper :—

Methane	0·1372
CO_2	0·8678
Volatile acids	1·0023
	2·0073

The fatty acids consisted mainly of acetic acid, together with smaller quantities of butyric acid.

3. The fermentation of cellulose above described takes place in absence of air. It is obvious, however, that much of the natural destruction of cellulose, *e.g.*, the mass of dead leaves which fall each autumn, must take place in presence of air.

Researches by Van Iterson have indicated various methods by which this breaking up can take place. He found that in presence of nitrates certain organisms are capable of oxidising cellulose, utilising the oxygen of the nitrate which is simultaneously reduced. The following experiment will illustrate this action :—

100 c.c. of tap water are placed in a 200 c.c. flask together with 2 grams of Swedish filter paper, 0·25 gram potassium nitrate, 0·05 gram potassium hydrogen phosphate (K_2HPO_4), a few c.c. of sewage and a little leaf mould. The flask is then filled to the neck, plugged with cotton wool and placed in an

incubator at 35° C. In fifteen days, if the conditions of the experiment are successfully realised, all nitrite and nitrate will have disappeared : 100 c.c. of the solution are then poured off and a further 100 c.c. of tap water, containing the same quantities of potassium nitrate and potassium hydrogen phosphate as originally used, are added. On incubating, the nitrate will be found to disappear much more rapidly, and on further repetitions of the process Van Iterson was able to reduce 0·5 gram potassium nitrate in one or two days ; the paper in the meanwhile disintegrates and disappears, and potassium carbonate or bicarbonate is found in solution. The evolution of nitrogen was observed, but no trace of hydrogen, methane or nitrous oxide. The equations representing this change are given by Van Iterson as follows :—

$$5C_6H_{10}O_5 + 24KNO_3 = 24KHCO_3 + 12N_2 + 6CO_2 + 13H_2O$$
$$C_6H_{10}O_5 + 8KNO_2 = 4KHCO_3 + 2K_2CO_3 + 4N_2 + 3H_2O$$

The evolution of gas during this reaction can be demonstrated, as in the case of the anaerobic decomposition of cellulose, by attaching a flask to the end of the Hempel gas burette, the flask being kept meanwhile in a constant temperature water-bath.

For the qualitative demonstration of the evolution of gas in decompositions of this sort, and to obtain a rough idea of its rate of evolution, it is only necessary to provide the evolution flask with a suitably stoppered inlet and outlet tube, the latter reaching nearly to the bottom of the flask, and being bent twice at right angles. As the gas is evolved, the liquid is pushed out, and can be measured from time to time, its volume obviously being equal to the volume of the gas evolved. At the end of the experiment the exit tube can be connected to a cylinder of water, and the gas in the original vessel drawn out by the inlet tube into a Hempel burette and examined, water entering to replace the gas. The arrangement has the disadvantage that

liquid is removed from the fermentation flask, and so the conditions of the experiment are altered as it proceeds. But if the fermentation is mainly confined, as in the case of cellulose, to the deposited matter at the bottom of the flask, the removal of the liquid is not of such serious moment, and it is convenient to have an apparatus the whole of which can be placed in an ordinary incubator.

Van Iterson found that the decomposition of cellulose in presence of nitrate, as above described, went on at much the same rate as the anaerobic change studied by Omelianski. He drew attention to the fact that the nitrite produced is readily re-oxidised by the organisms of nitrification, and consequently that in presence of air the nitrate is being continually reproduced, and the conditions for the destruction of cellulose are therefore constantly maintained. This observation is of great importance in connection with the destruction of cellulose in the bacterial filter beds employed in the purification of sewage.

Van Iterson found further that the spores and mycelia of higher fungi were also active in breaking down cellulose in presence of air. Thus, if a little leaf mould is placed in contact with moist filter paper in a moist chamber, rotting of the filter paper takes place with production in general of yellow stains. This is probably a complex process wherein various moulds together with chromogenic or pigmenting bacteria take part. It appears that woody fibre resists decomposition under these circumstances, and may remain practically intact for a long period of time in a disintegrated condition, in such end products as peat, lignite, etc. These contain the somewhat ill-defined substance known as *humus*, which is also formed by prolonged boiling of sugars with dilute acids. Humus bodies are generally of an acid character, dissolving in alkalis to form brown solutions.

The Decomposition of Pectose Bodies.—This fermentation has been studied by Wienogradski and his pupils, and

has been found to be due to an anaerobic bacillus, which will decompose pectin and calcium pectate, but has no action on cellulose. This fermentation is of great importance in connection with the retting of flax and other fibre. In such a process it is necessary to separate the fibres, which are held together by an integument consisting largely of calcium pectate. It is necessary to disintegrate this without injury to the fibre, and the object is best accomplished by a fermentation or retting process, which decomposes the integument while leaving the fibre intact.

CHAPTER XI

MISCELLANEOUS FERMENTATIONS, FAT-SPLITTING ENZYMES, OXIDASES, CLOTTING ENZYMES

Fat-Splitting Enzymes.—It is a matter of common observation that household fat, if allowed to accumulate, becomes what is termed rancid and evil smelling. This is due to fermentation of the fat with production, amongst other substances, of free fatty acids, which have an unpleasant smell.

Fats are defined chemically as esters of the so-called fatty acids with glycerine. Glycerine is an alcohol containing three hydroxyl groups with the formula $CH_2OHCHOHCH_2OH$.

Mutton or beef fat or stearin is a compound of glycerine and stearic acid, the latter having the formula $C_{17}H_{35}COOH$. Stearin therefore, being a glycerol ester of stearic acid, has the formula $C_3H_5(C_{17}H_{34}CO_2)_3$.

Soap is formed by the decomposition of fats by means of alkali, glycerine being obtained as a by-product, while the soap is the alkali salt of the fatty acid. Thus, e.g., if stearin is heated with caustic soda the following reaction takes place :

$$\begin{array}{l} C_{17}H_{34}CO_2{-}CH_2 \\ | \\ C_{17}H_{34}CO_2{-}CH \\ | \\ C_{17}H_{34}CO_2{-}CH_2 \end{array} + 3NaOH = \begin{array}{l} C_{17}H_{34}CO_2Na \\ | \\ C_{17}H_{34}CO_2Na \\ | \\ C_{17}H_{34}CO_2Na \end{array} + \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array}$$

Stearin — Caustic Soda — Sodium Stearate (Soap) — Glycerol

This process of splitting up of fat with formation of a soap

is known as the *saponification* of a fat. The term saponification has come to be a general one applied to all processes whereby a fat is split up, yielding a fatty acid and glycerine ; the process, indeed, is essentially one of hydrolysis and may be expressed in general terms in the following equation, where R = the residue of a fatty acid :—

$$R_3C_3H_5 + 3H_2O = 3RH + C_3H_5(OH)_3$$

Such a reaction can, e.g., be brought about by heating a fat with a mineral acid, or even by the action of steam under pressure.

Nature's method, however, for effecting this change, which is of primary importance in the assimilation of fat by living organisms, is as usual a much less drastic one. In the plant or animal which uses fat to build up its body substances, enzymes are produced known as *lipolytic* or *fat-splitting* enzymes, which are generally referred to as an individual enzyme under the term **lipase** or *steapsin*.

The decomposition of *animal* fats by lipase may be illustrated by taking *butter fat* as an example which is a compound of glycerine and butyric acid. This is readily obtained by melting a small quantity of butter in an evaporating dish over a water-bath and pouring off the liquid portion, leaving the solid residue of casein ; or more exactly by warming the butter with ether, filtering through filter paper, and distilling off the ether. The butter fat is a neutral yellow liquid, as can be ascertained by testing the ethereal solution with litmus paper.

To determine the action of lipase upon it *liquor pancreaticus* may be utilised. It was shown by Claude Bernard that digestion of fat was mainly brought about by pancreatic juice.

A few c.c. of butter fat may therefore be placed in a test-tube and thoroughly shaken with a few drops of ' liquor pancreaticus,' when an emulsion is formed. On warming this emulsion on a water-bath or incubator at 40° C. for some

hours and testing again with litmus the mixture will be found to have become acid.

A similar tube of butter fat incubated without the addition of the pancreatic extract will be found to be unchanged. If the action of the pancreatic juice is sufficiently prolonged, the peculiar unpleasant smell of butyric acid can be recognised.

In order to demonstrate the action of lipase upon a *vegetable* fat, *castor oil seeds* may be made use of. Just as the barley plant derives its nutriment from starch during the early stages of growth, and for that purpose secretes during germination an *amylase* which hydrolyses the starch in the grain, so the germ of the castor oil plant secretes a *lipase* which hydrolyses the oil (a glyceride of ricinoleic acid) contained in its seeds. To demonstrate this, therefore, castor oil seeds are allowed to germinate for some days by embedding them in moist sand placed in a small dish, which again can be placed in a moist chamber, and the whole incubated at a moderate temperature. When the seeds show signs of sprouting they may be thoroughly ground up in a mortar and the enzyme investigated in one of two ways.

1. The fat may be extracted by grinding up with ether and filtering, the operation being repeated several times till no more fat is extracted, as can be readily ascertained by evaporating a little of the ethereal solution on a watch glass : when the fat extraction is complete, no residue should be left.

This may also be effected by continuous extraction with ether in a Sohxlet apparatus, but it is probable that an extraction at the ordinary temperature gives a more active product ; in each case the ether is allowed to evaporate spontaneously in the air without heat, and the residue then contains the lipase.

2. The germinated seeds are ground in a mortar with a solution containing 5 per cent. of sodium chloride and 0·2 per cent. potassium cyanide, which is allowed to stand in contact

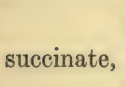
with the ground seeds for twenty-four hours. The solution is then filtered and can be tested for the presence of lipase.

To test for the presence of lipase, either in the ether extracted residue of the seeds, or in the solution obtained as described, an emulsion of castor oil is made by thoroughly shaking, say, 5 c.c. of the oil with a little gum arabic. Six test-tubes may now be made up as follows : to each of them 2 c.c. of a castor oil emulsion may be added together with a drop or two of neutral litmus, to two a few centigrams of the residue from the ether extraction of the seeds may be added, to two others, say, one c.c. of the sodium chloride extract, while the remaining two test-tubes are left as controls. One test-tube from each pair is now boiled, and after cooling all six test-tubes are incubated for some hours at a temperature of 35° C. In the case of the tubes containing the unboiled enzyme the formation of acid will be evident from the reddening of the litmus, while the boiled liquids, and the unboiled liquid to which no enzyme has been added, remain unchanged.

The actual amount of acid produced can be determined by adding dilute standard caustic soda, say $\frac{N}{100}$, till the blue colour of the litmus is restored.

Not only is lipase capable of splitting up fats properly so-called, but it can also decompose simpler esters, and the reaction in such a case, owing to the more complete solubility of the products, is capable of being more exactly studied. For this purpose ethyl butyrate, which has the formula $C_3H_7CO_2C_2H_5$, has been utilised by several investigators. Among these researches those of Armstrong and Ormerod in England and Kastel and Loevenhart in America may be specially mentioned. Armstrong and Ormerod made use of the dried residue obtained on extracting the castor oil with ether ; they found that the action of the lipase was increased by the presence of dilute acid. Their investigations were directed towards finding some chemical explanation of the action of the enzyme, and for this purpose they investigated a number

of esters both of mono-basic and of di-basic acids. They proposed a provisional hypothesis, according to which the hydrolysis of ethereal salts by lipase involves the direct association of the enzyme with the carboxyl group. Where these groups are close together hydrolysis appears to take place more readily than when they are separated; thus, ethyl



succinate, , is more readily broken up than ethyl



tartrate, , while ethyl malonate,  ,

occupies an intermediate position.

They concluded that the difference between animal and vegetable lipase is one of degree, and if sufficient enzyme is used almost all esters are more or less attacked.

Kastel and Loevenhart made use of animal lipase in the following way: they macerated fresh pancreas with coarse sand, extracted the enzyme with water or glycerine, 1 c.c. of the extract from either 10, 20 or 50 grams of tissue was diluted to 100 c.c. and allowed to act for forty minutes on a mixture of 4 c.c. of water, 0·01 c.c. toluene, and 0·25 c.c. of ethyl butyrate at 40°C., the mixture being afterwards titrated with $\frac{N}{20}$ potash solution. They found that the enzyme was destroyed at a temperature of 60° to 70° C., and that most antiseptics had an injurious effect on it, especially sodium fluoride and mineral acids.

By titrating the solutions at definite intervals of time they obtained results which led them to the following conclusions:—

1. The velocity of the reaction was not proportional to the amount of ester present.

2. The velocity of the reaction was nearly proportional to the concentration of the enzyme.

3. The reaction in general did not attain completion; only when a large quantity of enzyme was present in proportion to the ester was the decomposition of the latter nearly complete.

4. The coefficient of velocity of the reaction, that is, the ratio of decomposed ester to undecomposed ester per unit of time, was not constant but decreased with the progress of the reaction.

These results indicate that the reaction belongs to the class of changes known as reversible, and that there is a tendency for an equilibrium to be established between the action resulting in the decomposition of the ester, and the reverse action tending to combination of the free acid and alcohol. It will be remembered that a similar case was met with by Croft Hill when studying the decomposition of maltose, and Kastel and Loevenhart have added to the number of synthetic enzyme actions by effecting a synthesis of ethyl butyrate by the bringing together of ethyl alcohol and butyric acid in the presence of lipase.

Secretion of Lipase by Micro-organisms.—The secretion of lipase by micro-organisms can be demonstrated in a similar manner to the secretion of amylase, viz., e.g., by growing *Aspergillus niger* on a substratum of suet or butter. Moreover, if a little butter be melted in a Petri dish and allowed to set and some dilute sewage be poured over it, liquefaction and accompanying rancidity will soon be observable. These reactions are of importance in connection with the treatment of sewage by anaerobic processes.

The destruction of fat under *aerobic* conditions is very probably the work of higher organisms such as worms.

OXIDASES

It is again a matter of common observation that if, e.g., an apple is cut open and the interior is left exposed to air, in a short time it becomes brown. Everyone, too, must have been struck by the difference in appearance between mushrooms as bought in the shop and the same when freshly gathered; the dark brown appearance, especially of the under surface, is an unpleasant change from the delicate white and pink they exhibited whilst growing. These and many other similar changes are due to oxidation brought about by a class of enzymes known as **oxidases**; that the change is due to the presence of oxygen can be shown by leaving freshly-cut slices of apple in vacuo or in an inert atmosphere such as hydrogen, when no browning takes place.

Oxidases are very widely distributed enzymes, and for this reason a great many vegetable extracts and juices tend to darken on standing. A notable instance of such a change is the case of the juice of the lac tree, which furnishes the raw material of Japanese lacquer; this juice is a clear yellow when first drawn, exposed to air it rapidly turns brown and finally black. It has been discovered that this is due to an oxidising enzyme which has been termed **laccase.**

The browning of wine which takes place in course of time, and which is known as ageing, is due to the oxidation and precipitation of the colouring matter; this can be accelerated by the addition of an oxidase.

These enzymes have been studied in the same manner as other cases already considered; the following instances from numerous researches will serve to illustrate the methods employed. Laccase was investigated by Yoshida, who discovered in Japanese lac an acid, urushic acid, which is

capable of oxidation to the substance known as oxiurushic acid thus :—

$$2C_{14}H_{19}O_2 + 3O = 2C_{14}H_{18}O_3 + H_2O$$

Some ten years later Bertrand separated the juice into laccol, an alcohol derivative which was soluble in alcohol, and into the enzyme laccase which was insoluble in alcohol. Laccol was found to oxidise spontaneously, but the rate of oxidation was greatly accelerated when laccase was present.

An enzyme with the same properties was obtained from many vegetables, especially mushrooms of various sorts. The same enzyme also will oxidise numerous hydroxy and amido derivatives of benzene to quinone : thus in the case of hydroquinone the following reaction takes place :—

$$C_6H_4(OH)_2 + O = C_6H_4O_2 + H_2O$$

While the action of laccase, or an enzyme akin to it, is not specific, in the sense that one reaction and one only can be brought about by its intervention, yet it has its limitations, and will only oxidise such bodies as are capable of yielding quinols. It does not, therefore, oxidise tyrosin, the formula of which, it may be remembered, is

$$C_6H_4OHCH_2CHNH_2CO_2H$$

and which would therefore require to be broken up completely before a quinol could result from its oxidation. Tyrosin can, however, be oxidised by a specific enzyme known as **tyrosinase,** which had quite recently been investigated by Gortner. The source of Gortner's enzyme was the meal worm. To obtain the enzyme the larvæ were ground in a mortar with chloroform water, and the milky liquid strained through a cheese cloth : the milky extract if kept a short time in the air rapidly darkens on the surface, it remains white where not in contact with oxygen. It was found that a soluble and insoluble tyrosinase

was present in this extract: the soluble tyrosinase could be precipitated with ammonium sulphate from the filtrate left on filtering off the insoluble enzyme. The soluble enzyme was capable of colouring tyrosin dark violet black, with the final formation of a precipitate, within twenty-four hours; this reaction did not take place if the extract was previously heated to 90° C. The insoluble tyrosinase caused the tyrosin solution to undergo a series of colour changes ranging through pink, rose, violet and blue-black to a deposition of a black pigment-like substance, leaving the supernatant liquid completely decolourised. That a small quantity of the enzyme was able to affect a large quantity of tyrosin was proved by pouring away the colourless supernatant liquid and adding more tyrosin solution, when the series of colour changes was repeated. This operation of pouring off the colourless solution and adding more tyrosin was done seven times with one specimen of insoluble tyrosinase, weighing approximately 0·01 gram. Tyrosinase, therefore, has the property of continuous activity which is characteristic of enzymes in general.

The brown colour of **tea** is due to an oxidase formed in the growing leaf. This has been investigated by Mann, who extracted the enzyme in the following way :—

Ten grams of fresh, or 6·6 grams of the withered leaf, were ground to pulp, 5 grams of hide powder were added in order to precipitate the tannin, and the mixture ground together with a known quantity of water in which the enzyme is soluble. After standing two hours the mixture was pressed through a cloth and precipitated by alcohol, the amount of enzyme present in the solution could be determined by the intensity of the blue colour produced in a known amount of the solution by guiachum tincture; a certain proportion of the enzyme gave a blue colour with guiachum resin alone, while another portion required the addition of hydrogen peroxide before the blue colour was obtained. Only those

enzymes which give a blue colour with guiachum tincture without hydrogen peroxide are true oxidases.

THE CLOTTING ENZYMES

Rennet.—This enzyme, which is also sometimes referred to as *lab* or *chymosin*, has the property of curdling or clotting milk. It is generally prepared from the stomach of the calf; an impure product can be obtained by macerating the stomach with water or with a 5 to 10 per cent. solution of sodium chloride in presence of a little acid.

It is obtained in a purer state by digestion with sodium chloride solution 0·5 per cent. strength at 30° C. for twenty-four hours. On filtering the solution and adding acid up to 0·1 per cent. a precipitate of mucous matter is obtained which can be filtered off; acid is then further added to the filtrate up to 0·5 per cent., and the solution saturated with sodium chloride. On standing and stirring for two or three days and gradually raising the temperature to 30° or 35° C. a flocculent scum of rennet separates which is soluble in water.

An enzyme capable of clotting milk occurs in many animal and plant extracts, e.g., in germinating castor oil seeds; certain bacteria also secrete a clotting enzyme.

The chemical action of rennet upon milk is of considerable interest. On addition of rennet to milk a curd separates out, but the whey still contains an albumin, which differs from lact-albumin in that it is not precipitated by boiling. The curd also is different from the precipitate produced by acids, as this can be redissolved on neutralisation, while the curd produced by rennet is insoluble. It has been found that curd contains *calcium phosphate*, which is consequently present in cheese. If calcium phosphate is dialysed out of milk, curdling is no longer obtained by addition of rennet. It would appear that the greater part of the albumin of milk

exists as a body which may be termed *caseinogen*; the action of rennet is to break up this substance, a portion of which remains in solution, the remainder being precipitated, together with calcium phosphate, as *casein*. This theory of the action of rennet derives support from the following experiment: the caseinogen as a whole may be precipitated by acetic acid, washed and dissolved in lime water. On neutralising with phosphoric acid, a milky-looking liquid is obtained which forms a clot on addition of rennet.

Another method of exhibiting the same phenomenon is to dissolve the caseinogen in acid sodium phosphate, add the rennet to the solution and allow it to act for, say, half an hour. No clotting occurs; the solution is then boiled to destroy the activity of the enzyme; on adding calcium chloride, clotting at once takes place.

This last experiment, which was devised by Hammersten, would indicate that the action of the rennet is simply to break up the caseinogen, the subsequent clotting being due to calcium salts.

The addition of peptone to milk inhibits the clotting effect of rennet, probably owing to its affinity for calcium salts. It may be finally mentioned that the optimum temperature for the action of rennet is 40° C., while its activity is destroyed above 70° C.

Other important clotting enzymes are the **fibrin ferment** or **thrombose,** which causes the clotting of the blood, and **pectase,** which gelatinises fruit juices containing pectin; in both these cases, as in the case of rennet, calcium salts play an important part.

The investigation of the action of thrombase indicates that a substance which has been termed fibrinogen occurs in unshed blood. The action of thrombase is to precipitate fibrin, which carries down with it the red corpuscles of the blood, leaving a globulin dissolved in the clear serum. This phenomenon does not occur in the absence of calcium

salts, especially calcium sulphate. Thrombase can hardly be present in the free state in the body, but apparently must be looked upon as entering into combination with some other substance to form a zymogen, from which it is separated apart from the body in presence of calcium salts.

CHAPTER XII

OUTLINES OF THE CHEMISTRY OF ALBUMINS OR PROTEINS

All living organisms contain as an essential constituent a highly complex nitrogen-containing substance known generally as *protoplasm*. The simplest of all organisms, e.g., the amœba, is virtually a simple mass of protoplasm ; it has the property when alive of dividing into smaller living portions, and of building itself up from elements absorbed from its external surroundings.

The most highly developed animal, chemically considered, is a vast aggregation of cells of different structure and function, but all of them containing protoplasm in some form or other. Protoplasm is in no sense a chemical entity with a definite composition such as may be ascribed to even highly complicated organic substances ; it possesses structure visible under the microscope, and must be looked upon when alive as a constantly changing complex, wherein loose combinations are constantly being formed and decompositions taking place. Protoplasm may indeed be regarded as a factory where raw material of various kinds is taken in, where finished products are delivered, and where a certain amount of waste material is produced.

It would obviously be of little help to the understanding of the operations of such a factory simply to know the materials of which it is composed, or even the bare enumeration of its contents in terms of iron and steel and bricks and mortar or

weight of stores. The ultimate chemical composition of protoplasm, therefore, can tell us little of its real nature; it is of interest to know that its invariable constituents include carbon and nitrogen, and almost universally phosphorus. It will obviously be more instructive to describe it as it were by stages, classifying the chemical contents into substances of gradually decreasing complexity. Even then we shall only have obtained a vague idea of the constituents of dead protoplasm, as we might make an inventory of the contents of our hypothetical factory after business had been shut down. Of the course of operations, or the economic conduct of the factory, we should know little or nothing. Having obtained such an inventory, however, and presuming the factory began work again, by taking careful note of the material entering and leaving the factory, we could form a much better idea of the nature of the processes carried on therein. The task which confronts the chemist is to investigate in this kind of way the chemistry of protoplasm, which in other words is the chemistry of life. In the present chapter an attempt will be made broadly to indicate essential facts with reference to the products of the activity of protoplasm. The substances which have been isolated as more or less definite chemical entities belong to the class known generally as *albumins*, *proteid* bodies, or more recently as *proteins*.

It will probably be simplest to take one or two of the most characteristic of these substances and study their properties and products of decomposition; afterwards will be given in brief summary an account of the principal bodies of this class which are known, together with their decomposition products. At the same time occasion will be taken to indicate certain of the main lines of investigation which are at present being made use of in regard to them.

As a typical albumin ordinary *white of egg* may be made use of, and the following experiments carried out:—

Experiment.—About 1 c.c. of white of egg may be poured

into 50 c.c. of water, stirring meanwhile; a white precipitate is formed. This can be filtered off and a portion of the filtrate boiled, when a further precipitate is obtained.

It will thus be seen that the egg-white can be separated readily into two substances, one soluble, the other insoluble, in cold water. The insoluble portion is known as *globulin*, the soluble substance is *albumin*.

As the word albumin is also used in a more or less generic sense, it is better perhaps to refer to this body as *egg-albumin*. A related substance can be obtained from blood serum and is known as *serum albumin*, and also from milk, when it is known as *lact-albumin*.

A larger quantity of egg-albumin solution may now be prepared by adding further quantities of egg-white to water, stirring, and filtering off the globulin; the solution of egg-albumin can then be used for investigating certain typical properties of this class of substance. In the first place a number of simple qualitative tests may be carried out, which will indicate the presence of certain elements in albumin, and the class of chemical substances to which it may be referred.

Experiment.—5 c.c. of the solution may be warmed with a little strong caustic soda; an evolution of *ammonia* can readily be detected which indicates the presence of *nitrogen*, and further that it is most probably present, in part at least, in combination as a so-called amino group or NH_2.

Experiment.—A few drops of lead acetate are added to a 20 per cent. solution of caustic soda; a precipitate is formed which readily redissolves. If a little of this solution is boiled with a solution of egg-albumin, it rapidly darkens owing to the formation of sulphide of lead; this indicates the presence of *sulphur* in the egg-albumin.

Experiment.—On warming with strong caustic soda and adding a few drops of dilute copper sulphate solution a violet colour is obtained. This is known as Piotrowski's reaction. If

the boiling with caustic soda is prolonged, a rose-pink colour is obtained on addition of copper sulphate, i.e., the *biuret* reaction. This has been already observed, as a characteristic reaction for enzymes, when examining the properties of amylase. The formation of this colour is due to the production of biuret or an allied substance; the biuret group, it will be noted, therefore is characteristic of the decomposition products of albumin, and the biuret reaction is a useful indicator of the extent to which decomposition has taken place.

Experiment.—The *Xanthoproteic reaction*, i.e., the orange colour obtained on warming with strong nitric acid and subsequently adding ammonia, is a general reaction for albumins as they are broken up by the strong nitric acid.

Experiment.—Millon's reagent will be found to give a brick-red precipitate on boiling with the albumin solution.

It will already have been noticed, and it is of course common knowledge, that egg-albumin is coagulated on heating; this would suggest that egg-albumin belongs to the class of substances known as *colloids*. This can be demonstrated by enclosing a solution of albumin in a parchment cylinder, adding a little thymol to prevent putrefaction, and immersing the cylinder in water. On boiling a portion of the external water at intervals no coagulation will take place; the albumin will be found still present in the interior of the parchment cylinder, and capable of coagulation.

Like other colloids, egg-albumin can be precipitated by the addition of certain salts. Thus ordinary sodium chloride, magnesium sulphate, zinc acetate, may be employed for this purpose, and especially ammonium sulphate.

The chief methods in use for the separation of albumin substances consist in fractional precipitation by means of certain salts.

It is possible to obtain certain albumins in an approximately crystalline state. Egg-albumin may be taken as an example. The following description of the method of pre-

paration of crystalline albumin is taken, with some modification, from the monograph on ' The General Characters of the Proteins ' by Dr. Schreyver, p. 20.

Egg-white is beaten to a froth (to break up the membranes) with exactly its own bulk of saturated ammonium sulphate solution. The mixture, after standing overnight, or at least for some hours, is filtered from the precipitated globulin. The filtrate is now measured. Ten per cent. acetic acid (glacial acetic acid diluted to ten times its bulk) is then very gradually added from a burette, until a *well-marked* precipitate forms. The object of the addition of acid is to neutralise the alkalinity which is developed in the ammonium sulphate solution on standing. The formation of a precipitate indicates the point of neutralisation. A further quantity of acid is now added, 1 c.c. for each 100 c.c. of the filtered mixture as already measured. A bulky precipitate is thus produced, which is at first amorphous, but which becomes crystalline in the course of four or five hours, if shaken from time to time. To obtain the full yield, the material should stand for twenty-four hours. The precipitate can then be filtered off, and allowed to drain on a plate of porous porcelain. The precipitate will probably contain ammonium sulphate, from which indeed it is not easy completely to free it ; but it can be obtained in a purer state by redissolving in water, adding half-saturated ammonium sulphate, containing acetic acid in the proportion of 1 per 1000, till a permanent precipitate forms, and finally a further 2 c.c. of ammonium sulphate in excess of this.

Albumin substances belong as a rule to the class of compounds known as *amphoteric*, that is, they are capable of acting both as weak acids and as weak bases. A solution of albumin in dilute alkali is sometimes known as *alkali-albumin*. If acid is added very carefully to such a solution, the albumin is first precipitated and then redissolved, forming so-called

acid-albumin. Thus albumin, it will be seen, is capable of forming salts and of combining both with acids and bases.

Hydrated oxides such as aluminium hydroxide, $Al_2(OH)_6$, or ferric hydroxide, $Fe_2(OH)_6$, are capable of precipitating albumin from solution. This phenomenon is no doubt partly physical and partly chemical; physical in that one colloid body on separating from solution tends to attract other colloids by a process known as *adsorption*; and chemical in that the metallic hydroxide actually combines with the albumin. This property by which colloidal precipitates tend to carry out of solution other colloids, especially those related to albumin, finds application on the large scale in the chemical precipitation of sewage and other polluted liquids.

The well-known household cookery receipt for clarifying soup, etc., by means of white of egg is an illustration of the same property.

It has been pointed out by the writer of this book and others, that by carefully conducted precipitation, either with hydrated alumina or ferric hydroxide, it is possible to remove from solution all colloidal matter, and to obtain results similar to those which are obtained by dialysis. The method may be illustrated by the following example of what has been termed the *clarification test.*

Two hundred cubic centimetres of the liquid to be examined, e.g., a sample of sewage (freed from grosser solid matter by settlement and decantation), or a solution containing albumin, is treated with 2 c.c. of 10 per cent. solution of iron or aluminium alum, together with 2 c.c. of a 10 per cent. solution of sodium acetate, and boiled vigorously for two minutes; on cooling and filtering through filter paper a crystal clear solution is obtained. By making a suitable analytical estimation, e.g., of the amount of oxygen absorbed from an acid solution of potassium permanganate of known strength, or by boiling a known amount of the clarified and unclarified liquid respectively with alkaline perman-

ganate and determining the so-called ' albuminoid ammonia ' evolved, a measure is obtained of the quantity of albumin substance removed from solution. It should be noted, of course, that in a complicated substance like sewage other substances besides albumins, notably e.g. fats, are carried down by this process.

Besides hydroxides of aluminium and iron, hydrated copper oxide combines readily with albumin, and copper salts have been used on a large scale in the treatment of water supplies, more especially with the object of preventing the growth of algae in reservoirs. It is probable that the toxic action of copper in this respect, and also its analogous action as a germicide, is due to the readiness with which insoluble compounds of copper and albumin are formed.

Albumin can be recovered from its compounds with metallic oxides by treatment with acids, when the metal goes into solution and the albumin is precipitated. By careful treatment of a copper compound it has been possible to obtain albumin in a form which is not crystalline, which is almost completely soluble in alcohol and which does not coagulate on boiling. The following description is given by Harnack (*Ber.* XXII. ii. pp. 30–46) : A clear solution of albumin is obtained by dissolving egg-albumin in water and filtering off the globulin ; acetic acid is added and the precipitate obtained filtered off. The filtrate is exactly neutralised and again filtered ; in this way the remaining portions of globulin are removed. The neutral solution is now precipitated with copper sulphate and the precipitate thoroughly washed, then suspended in water, dissolved in a *few drops* of caustic soda and reprecipitated with acetic acid. The precipitate is again washed, redissolved in caustic soda and precipitated with acetic acid, and again thoroughly washed. It is then dissolved in *excess* of caustic soda and the dark violet-blue jelly allowed to stand twenty-four hours, when it is precipitated with hydrochloric acid, the copper in this case going into

solution. The precipitated albumin is carefully washed on a filter pump, and finally dried in a platinum dish at a temperature not exceeding 100° C. It is necessary in this process to use plenty of material to start with, as the losses by washing, especially in the final removal of the copper, are apt to be considerable. The preparation, however, is of much interest as affording a means of obtaining albumin in a pure state and in a form more convenient for investigation than that in which it is commonly found.

In the foregoing paragraphs the properties of a typical albumin have been considered in some detail, apart from the study of the products obtained when it is submitted to partial decomposition. This study may now be followed up, keeping always to the one typical substance, viz., egg-albumin. In the light of the information thus obtained it will be easier to follow the subsequent general description of other substances of a similar nature.

It has already been shown by qualitative examination that on violently attacking albumin by such substances as strong caustic soda, the presence of end products such as ammonia, biuret and sulphuretted hydrogen could be detected. It is obvious, however, that such a procedure gives us but little information. Determinations by physical methods would indicate that the molecular weight of albumin is probably somewhere in the neighbourhood of 15,000. Its composition, according to ultimate analysis, can be expressed within the following limits :—

Carbon	50	to 55	per cent.
Hydrogen	6·9	„ 7·3	„
Nitrogen	15	„ 19	„
Oxygen	19	„ 24	„
Sulphur	0·3	„ 2·4	„

The information given by these figures is the same kind of information that would be obtained in regard to the construc-

tion of a watch, if it were stated to be made up of a certain weight of glass, of silver, of gold, of brass and of steel, together with a few precious stones. It is obviously necessary that, in order to get some idea of the construction of the watch, it must be taken to pieces carefully and each independent portion separately described. Similarly, in order to obtain even an approximate idea of the structure of the albumin molecule, means must be found to take it to pieces gradually, and to identify the products thus obtained. In order to accomplish this two means are at our disposal, viz., in the first place the action of acids, in the second place, and especially, the action of so-called *proteolytic enzymes*, that is, enzymes which are capable of breaking up protein substances. Of these the two chief are *pepsin* and *trypsin*. The methods of preparation of these and their characteristic modes of action may now be usefully considered.

Pepsin.—This is a characteristic enzyme of the gastric juice. Ordinary ' liquor pepticus ' is prepared by macerating the mucous membrane of the stomach of a dog or pig with dilute hydrochloric acid, 0·2 per cent., and filtering the solution. The filtered solution contains pepsin.

By extraction with glycerine in absence of acid a purer but less active product is obtained. The enzyme can be further purified by precipitation with sodium phosphate and calcium chloride, the calcium phosphate formed carrying down the enzyme. The enzyme is separated from the precipitate by solution in hydrochloric acid, and the mineral salts removed by dialysis, the salts passing through the parchment membrane, leaving a solution of the enzyme in the dialyser.

Trypsin.—Trypsin is the enzyme of the pancreatic juice and is obtained in a similar manner to pepsin, by digesting pancreatic tissue with dilute acid or glycerine at 35° to 40° C. The preparation of the pure enzyme is an exceedingly complex process.

The characteristic difference between pepsin and trypsin is that pepsin acts in dilute *acid* solution, and trypsin in dilute *alkaline* solution. The following experiments may usefully be made to illustrate the characteristic properties of these enzymes.

A quantity of hard-boiled egg-white may be cut up into strips of approximately 2 cm. × 5 mm. × 1 mm. dimensions and one of these placed in each of eleven test-tubes, to which the following additions are made in order, about 10 c.c. of solution being taken in each case :—

1. Water;
2. Hydrochloric acid,[1] 0·2 per cent.;
3. Water + ½ c.c. of 'liquor pepticus';
4. Hydrochloric acid, 0·2 per cent. + ½ c.c. 'liquor pepticus';
5. Hydrochloric acid, 0·2 per cent. + ½ c.c. 'liquor pepticus';
6. One per cent. sodium carbonate solution;
7. One per cent. sodium carbonate solution + ½ c.c. 'liquor pepticus';
8. Hydrochloric acid, 0·2 per cent. + ½ c.c. 'liquor pancreaticus';
9. Water + ½ c.c. 'liquor pancreaticus';
10. Sodium carbonate solution 1 per cent. + ½ c.c. 'liquor pancreaticus';
11. Sodium carbonate solution 1 per cent. + ½ c.c. 'liquor pancreaticus.'

All of these are now placed in a water-bath at 40° C., with the exception of numbers 5 and 11, which are boiled.

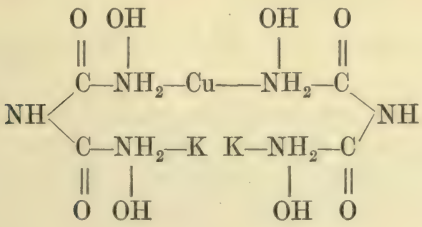
At the end of some hours the following results will be observed: the strips of egg-white will be virtually unattacked either by water, by dilute acid or alkali, by pepsin and alkali together, or by trypsin and acid together. On the other hand, some digestion will probably be observed in the case of both

[1] 110 c.c. of $\frac{HCl}{10}$ made up to 200 c.c. gives a solution of this strength.

pepsin and trypsin alone, while in the case of the mixture of pepsin and acid, and of trypsin and alkali, digestion will be almost complete.

This demonstrates the fact that pepsin is most active in presence of dilute acid, while trypsin is most active in the presence of dilute alkali. In order to investigate the products of decomposition in each of these cases larger quantities of egg-white must of course be taken ; if this is done, the products present in solution can then be investigated in the manner to be described.

In following the reaction it will be advisable to make observations from time to time, as the reaction is progressive, products of decreasing complexity being obtained as it proceeds. If to a portion of the solution shortly after the beginning of the reaction strong alcohol or a saturated solution of ammonium sulphate is added, a precipitate is formed ; the substances thus precipitated are known as **albumoses.** At a further stage ammonium sulphate is added ; no precipitate will be obtained, but a precipitate will still be formed if alcohol is added. These products of decomposition of albumin, which are soluble in water and precipitated by alcohol but not by ammonium sulphate, are known as **peptones.** It will be found on testing that they still give the biuret reaction, showing that a complex residue containing amino (NH_2) and imino (NH) groups is still present. The red substance of the biuret reaction is believed by Schiff to be a copper potassium compound having the following constitution :—



The further decomposition of peptones results in the formation first of substances which have still a complicated composition, and which are known as **polypeptides,** and finally into substances of a simpler character, viz., **amino acids,** of which amino-acetic acid or *glycocoll*, CH_2NH_2COOH, is a prototype. The separation and investigation of these is a task for the experienced organic chemist, and its details cannot profitably be fully discussed here.

The whole subject has been brilliantly investigated by Emil Fischer and his colleagues, who have not only devised methods for separating and identifying amino-acids, but have also been enabled to synthesise a number of polypeptides, whose complexity approaches in certain cases the complexity of the peptone molecule, and which are even capable of being broken down again into simpler substances by the action of trypsin.

Certain American investigators have announced that they were able to synthesise peptone-like bodies by the action of trypsin on polypeptides. However this may be, it is clear that in this direction we must look for any definite knowledge as to the ultimate structure of the albumin molecule or its derivatives, and a brief account of the chief products separated or prepared by Emil Fischer and others, and of the methods used in their researches, will be of interest and value as affording a basis for the classification of the very numerous bodies related to albumin.

PRIMARY DISINTEGRATION PRODUCTS OF ALBUMIN

Fischer made use of three chief methods for separating amino-acids :—

1. The acids are converted into ethyl esters which are separated by fractional distillation under the lowest possible pressure. The following description will indicate in outline the practical carrying out of the method.

The solution containing the mixture of amino-acids is carefully evaporated at reduced pressure and at a temperature not exceeding 40° C. The syrupy residue is dissolved in absolute alcohol, and gaseous hydrochloric acid passed into the solution to saturation, the hydrochlorides of the esters being thus formed. The excess of alcohol is evaporated off under diminished pressure. Strong caustic soda solution is carefully added to the residue, until the hydrochloric acid is neutralised.

The esters thus set free are separated by solution in ether. The etherial solution is then fractionally distilled in a specially designed apparatus in which the pressure is reduced to less than 1 mm.

A number of precautions in detail are necessary if the best yields are to be obtained.

2. The acids are converted into their β-naphthalene-sulpho derivatives, which are sparingly soluble compounds.

The following equation indicates the formation of the β-naphthalene-sulpho derivative of *serin* by the action of β-naphthalene-sulpho-chloride :—

$$C_{10}H_7SO_2Cl + H_2NCH\begin{matrix}CH_2OH\\ \diagdown\\ \diagup\\ COOH\end{matrix} = HCl + C_{10}H_7SO_2NHCH\begin{matrix}CH_2OH\\ \diagdown\\ \diagup\\ COOH\end{matrix}$$

3. The acids are combined with phenyl isocyanate, which gives characteristic compounds.

The equation representing the formation of the glycocoll compound is as follows :—

$$C_6H_5{-}NCO + NH_2CH_2COOH = C_6H_5NHCONHCH_2COOH$$

The chief end products obtained by taking to pieces, as it were, the molecule of albumin, may be roughly classified as follows :—

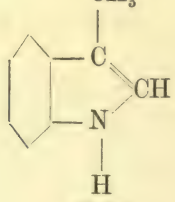
Mono-amino acids ;
Hydroxy-amino acids ;
Di-amino acids ;
Amino-dicarboxylic acids ;
Sulphur derivatives ;
Purin bases ;
Ptomaines ;
Carbohydrates.

In addition, simple substances such as sulphuretted hydrogen, carbonic acid, ammonia, sundry fatty and other acids, etc., are produced.

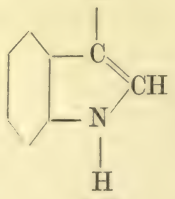
Mono-amino Acids

The following are the chief mono-amino acids :—

1. *Glycocoll or amino-acetic acid.*—This is the simplest member of the mono-amino acids ; it is frequently termed *glycin* for the sake of convenience in describing its numerous derivatives among the polypeptides. Glycocoll is related to what is believed to be the mother substance of *skatol*, a substance occurring in excreta, the unpleasant smell of which is largely due to it. Skatol can be recognised in concentrated fresh sewage by the pink colour which is obtained on warming with strong sulphuric acid. It has been shown to be β-methyl-indol, the relation of the two bodies skatol and indol being given by the following formulæ :—

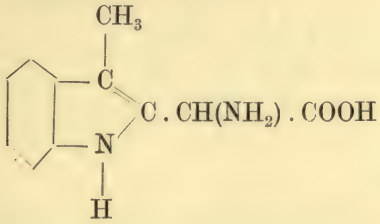


Skatol



Indol

It probably occurs as a decomposition product of albumin, in the form of skatol-amino-acetic acid :—



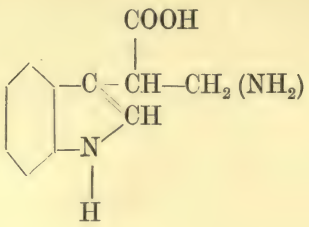
2. *Alanin or amino-propionic acid,* CH_3CHNH_2COOH. This acid has been shown by Emil Fischer to be widely distributed as a decomposition product of albuminoids. Its derivatives have many of them been well known for some time, especially *phenyl-alanin,* that is $C_6H_5CH_2CHNH_2COOH$, and hydroxy-phenyl-alanin, more commonly known as *tyrosin,*



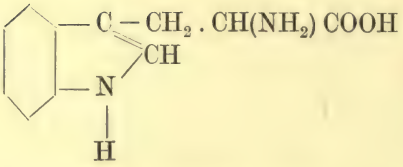
CH_2CHNH_2COOH

Tyrosin is easily isolated on account of its sparing solubility ; it is one of the products of excretion of the animal body and occurs together with *leucin.*

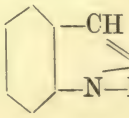
Other derivatives of alanin give rise to the very important substances indol and skatol already mentioned. A substance termed *tryptophane* has been isolated from the mixture of substances produced by the action of trypsin on albumin. Some amount of discussion has taken place as to the constitution of this body ; it appears certainly to be an indol-amino-propionic acid. Indol-amino-propionic acid or tryptophane will therefore have either of the two following formulæ :—



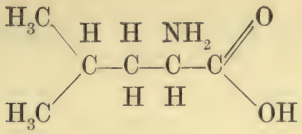
or



In this way we can see how indol may be produced by the decomposition of albumin substances. It is of interest to note that the formation of indol, recognised by the red coloration it produces with nitrous acid, is a characteristic reaction for certain bacteria, notably *B. coli*, and serves to distinguish this from the more dangerous typhoid bacillus. The *cholera-red* reaction given by the cholera organism depends also on the formation of indol.

In both cases the red coloration is due to the formation of nitroso-indol,  , formed by the action of nitrous acid on the *imino* group present.

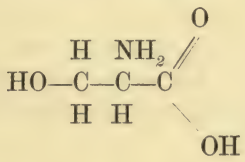
3. *Amino-valerianic acid*, $CH_3CH_2CH_2CHNH_2COOH$. This acid is of interest mainly on account of its derivatives, the chief of which is known as *arginin*, to which reference will be made later, and *leucin* or isobutyl-*a*-amino-acetic acid, which has the following formula :—



This is one of the earliest known of the decomposition products of albumin, and readily crystallises in scales or nodules with very characteristic appearance.

Hydroxy-amino Acids

Serin, *a*-amino-*β*-hydroxy-propionic acid,

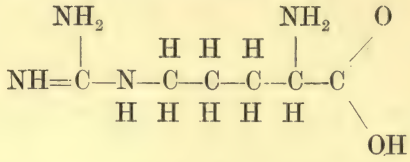


This acid is of special interest as being one of the chief decomposition products of silk. Emil Fischer has shown that it is a general product of the breaking up of albumin.

Di-amino Acids

There are three important di-amino acids which, according to Kossel, occur in all albumins in greater or less amount, and whose relative preponderance can therefore serve as a means of classification of albumin bodies. These three di-amino acids are termed by Kossel *hexone* bases, as they all contain six carbon atoms, and the basic character which is characteristic of all amino acids predominates. These three substances are *arginin*, *lysin*, and *histidin*. *Arginin* is the

guanidine-*a*-amino-valerianic acid; guanidine has the formula $NH{=}C(NH_2)_2$, and arginin, therefore, may be written



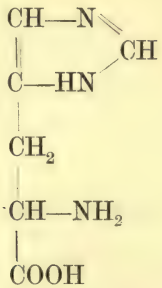
Arginin is really a compound of guanidine with *ornithin* a derivative of which is found in the urine of birds. Ornithin is *a*-δ-di-amino-valerianic acid.

Lysin is *a*-ϵ-di-amino-normal-caproic acid, i.e.

$$CH_2NH_2C_2CH_2CH_2CH_2CHNH_2CO_2H$$

It occurs in greatest quantities in casein and gelatine.

Histidin has a rather more complex constitution than either of the other two hexone bases. It is probably a condensation product formed by elimination of NH_3 from arginin and its constitutional formula may be provisionally written thus :—

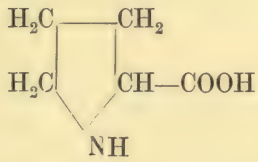


Amino-dicarboxylic Acids

Of these the following may be mentioned, *aspartic* acid or amino-succinic acid, $\begin{matrix}CHNH_2CO_2H\\ |\\ CH_2CO_2H\end{matrix}$, and *glutaminic acid* or *a*-amino-glutaric acid, $COOHCHNH_2CH_2CH_2CO_2H$.

Pyrollidin-carboxylic Acid or Prolin

This has been obtained as a product of hydrolysis of casein and is of interest from the point of view of the synthetical experiments of Emil Fischer and others. It has the following constitution :—



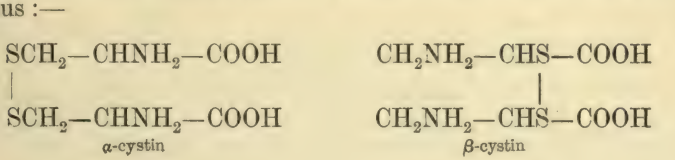
Sulphur Bodies

Cystin.—This is a very interesting substance as it is probably the parent body of the isomeric forms *α*-cystin and *β*-cystin, which are very likely the parent bodies of the unpleasant smelling sulphur derivatives of albumin. These two bodies are differentiated according to the products obtained when they are treated with hydrochloric acid under pressure, as indicated by the following formulæ :—

$$\underset{\alpha\text{-cystin}}{CH_2SH-CHNH_2-COOH} \rightarrow \underset{\alpha\text{-alanin}}{CH_2-CHNH_2-COOH} + \underset{\text{hydrogen}}{H_2S}$$

$$\underset{\beta\text{-cystin}}{CH_2NH_2-CHSH-COOH} \rightarrow \underset{\alpha\text{-thiolactic acid}}{CH_3-CHSH-COOH} + \underset{\text{ammonia}}{NH_3}$$

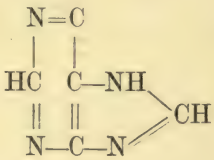
Probably both *α*- and *β*-cystin contain at least two groups as given in the above equations, joined in each case by sulphur thus :—



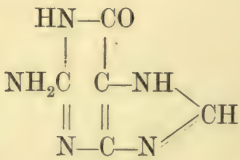
Purin Bases

These important substances are obtained as decomposition products of nucleic acid, produced in its turn from so-called nucleo albumins. They are derived from a parent substance, prepared by Emil Fischer, which he termed *purin*. The relation of the purin bases to purin is shown by the following formulæ :—

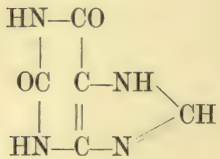
Purin .. $C_5H_4N_4$ =



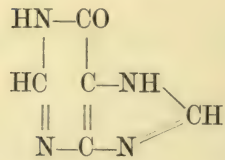
Guanin .. $C_5H_5ON_5$ =



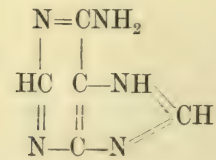
Xanthin .. $C_5H_4O_2N_4$ =



Hypoxanthin $C_5H_4ON_4$ =



Adenin .. $C_5H_5N_5$ =



Ptomaines

These bodies are products of putrefactive decomposition of albumin and are mostly strong bases; they can be obtained by splitting off CO_2 from amino acids. Thus leucin gives rise in this way to pentamethylene-diamine or *cadaverin* according to the following equation :—

$$CH_2NH_2-(CH_2)_3-CHNH_2COOH = CH_2NH_2-(CH_2)_3-CH_2NH_2 + CO_2$$

while argenin gives rise to putrescin, cyanamide being formed at the same time :—

$$NH_2C(NH)NH-CH_2-(CH_2)_2CHNH_2-COOH = \underset{\text{Cyanamide}}{NH_2-CN} + CO_2 + \underset{\text{Putrescin}}{NH_2CH_2-(CH_2)_2-CH_2NH_2}$$

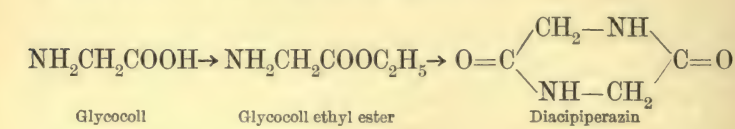
Carbohydrates

These occur among the decomposition products of certain albumins in the form of amino derivatives of which *glucosamin*, $CH_2OHCHOHCHOHCHOHCHNH_2CHO$, is a characteristic example.

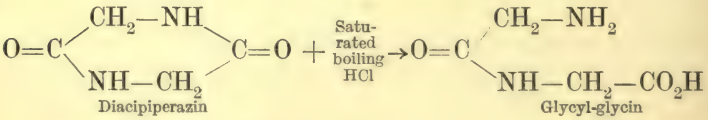
Synthesis of Disintegration Products.—We are now in a position to understand something of the significance of the syntheses of the complicated bodies known as **polypeptides,** from the starting point of the disintegration products which have just been described. It would lead too far to attempt to give these in any detail, but the simplest case will suffice to indicate the principle on which more complex substances may be built up. Glycocoll or glycin may be taken as a starting point.

The ethyl ester is first prepared; on standing, condensation

takes place, with formation of a ring compound known as *di-aci-piperazin*, or di-glycocoll anhydride,



On saturating a boiling solution of this compound with gaseous hydrochloric acid, it is split up with formation of the simplest polypeptide, known as *glycyl-glycin* or $NH_2CH_2CO-NHCH_2COOH$, the group NH_2CH_2CO being termed *glycyl*. The reaction is expressed as follows :—



It is readily seen that if glycyl-glycin is taken in its turn as a starting point, and a similar set of reactions carried out, further similar complexes of higher molecular weight could be obtained. The most complex polypeptide so far synthesised has the constitution :—

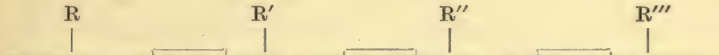
$$\left[\begin{matrix}NH_2CH(C_4H_9)CO[NHCH_2CO]_3NHCH(C_4H_9)CO[NHCH_2CO]_3\\ NHCH(C_4H_9)CO[NHCH_2CO]_8NHCH_2COOH\end{matrix}\right]$$

It is termed *l*-leucyl-triglycyl-*l*-leucyl-triglycyl-*l*-leucyl-octaglycyl glycin.

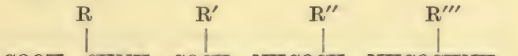
It is an *octadecapeptide*, containing no less than 18 amino-acid residues, giving it a molecular weight of 1213.

Compounds such as this give the biuret reaction, and are capable of being partially split up by ferments, such as trypsin ; they are in fact nearly akin to peptones, which, as we have seen, are some way on to the complexity of albumin.

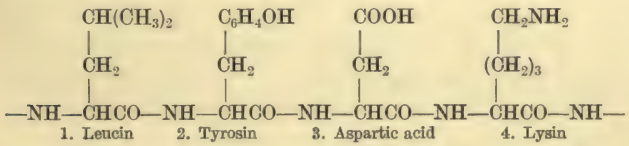
The Constitution of Albumins.—The investigation of the properties of the amino acids, the synthetical work of Fischer on the polypeptides, and other researches in similar directions, have led to the conception of the albumin molecule as consisting of a complex of amino-acid residues, linked together by the condensation of *a*-amino groups with carboxyl groups. The following complex will serve to illustrate the theory which has been propounded by Hoffmeister :—



On condensation this yields—



The groups R, R′, R″, etc., represent various residues which, on splitting off, give the various characteristic decomposition products of albumin. Thus, the following typical examples will serve for illustration :—



It can easily be seen how by simple hydrolytic changes the various substances leucin, tyrosin, aspartic acid, or lysin can be split off from such a complex. On oxidation with permanganate, these side chains are finally converted into oxalic acid and ammonia.

In ordinary animal metabolism, hydrolysis and oxidation go on together, with formation of urea as an end product. The constitution of individual albumins is by no means

sufficiently well known to permit of a strict chemical classification according to their decomposition products. An attempt has, however, been made by Kossel, who divides albumins into four classes, according to their yield of the so-called hexone bases already referred to, viz., lysin, arginin, and histidin. Kossel's classification was as follows :—

1. *Protamins*—All rich in arginin, but differing in the amounts of other bases and of mono-amino acids.
2. *Histones*—Relatively high in arginin.
3. *Vegetable albumins*.—Poor in arginin and no lysin.
4. All others containing all three hexone bases and most amino acids.

The Separation and Extraction of Albumins.—It has already been seen when studying the properties of ordinary egg-albumin that it was possible to separate it, e.g., from the associated substance globulin, by the insolubility of the latter in water. Further, it was found that whereas albumoses were precipitated by both alcohol and ammonium sulphate, peptones were precipitated by alcohol, and not by ammonium sulphate. The method of precipitation by suitable salts and other substances, if carried out with care, can be used for separating the various albumins one from another. Such a process is known as *salting out*.

The salts chiefly used for separation of the albumins are as follows, beginning with the least effective :—

Class I.	Sodium chloride ;
	Sodium sulphate ;
	Sodium acetate ;
	Sodium nitrate ;
	Magnesium sulphate.
Class II.	Potassium acetate.
Class III.	Ammonium sulphate ;
	Zinc sulphate.

The members of each class are more or less equivalent in precipitating power, but whereas, e.g., sodium chloride will not precipitate egg-albumin, ammonium sulphate will not only precipitate egg-albumin, but also its primary disintegration products, viz., albumoses.

In making salting-out experiments it is important that the concentration of the albumin solution shall not be altered. Thus, for example, to study the effect of various concentrations of any salt on an albumin solution, a number of test-tubes, each containing 2 c.c. of the albumin solution, may be taken, and 8 c.c. of a mixture, in varying proportions, of distilled water and a saturated solution of the salt under observation.

By experiments of this sort it has been found that the operation of salting out is subject to the following well-defined laws:—

1. The degree of concentration of any salt necessary for the precipitation of any particular albumin is characteristic for that body. If, for example, a serum solution is precipitated with ammonium sulphate, it has been found that the globulin begins to come down when ammonium sulphate is present to the extent of 24–29 per cent. of complete saturation. The albumin does not begin to be precipitated until the degree of saturation reaches about 64 per cent.

2. If one albumin is precipitated by a lower degree of concentration than others of any given salt, a proportionally lower concentration will also be effective with other salts.

Thus, in the example just given, if zinc sulphate were used instead of ammonium sulphate, less of it would be required to precipitate the globulin than the albumin.

3. The limits between which precipitation commences and finishes on addition of a salt to a solution are numbers characteristic for each albumin. Thus the precipitation of

globulin in a serum solution by means of ammonium sulphate begins at 24–29 per cent. of complete saturation, and is completely thrown out of solution when the saturation reaches 46 per cent. The corresponding limits for serum albumin are 64 and 90 per cent. of saturation.

In addition to the salts above mentioned, albumin can be precipitated, as we have seen, by colloidal metallic hydroxides.

Albumins also combine with numerous organic colouring matters, and advantage is taken of this in the various methods for staining tissues for microscopical examination. Many of the naturally occurring colouring matters exist in combination with albumin, from which they have to be separated if the pure colouring matter is required. In the indigo plant, for example, a portion of the indigo probably occurs in combination with indigo-gluten ; and there is evidence that laccainic acid, the colouring matter of lac dye, exists in the body of the lac insect as an insoluble albumin compound. These facts have their practical importance in connection with dyeing. The reason that wool can be dyed with certain colouring matters which are not taken up by cotton, that is by cellulose, is that wool is chemically related to albumin, and is therefore capable of combining with colouring matters, more especially those of an acid character. Further, various albumins, especially, e.g., serum albumin, as being obtainable in large quantity from the blood of slaughtered animals, is used as a mordant for fixing certain colours in calico printing.

For the precipitation of *peptones*—and to these may be added enzymes, which we have seen have many of the properties of peptones and are allied to them in composition—substances such as phosphotungstic and phosphomolybdic acids may be used. Metaphosphoric acid, and also a mixture of potassium ferrocyanide and concentrated acetic acid, can also be used for precipitation of bodies of this class ; it may be

remembered that metaphosphoric acid was used for the precipitation of the amylase of saliva.

Tannic acid also precipitates peptone bodies, and it is probable that the difficulty of extracting certain enzymes from plants depends on the fact that they exist in the plant in combination with tannic acid. It was for this reason that Brown and Morris, in their research on the amylase of foliage leaves, obtained better results by using powdered dry leaf than by using a watery extract.

In order to illustrate the preparation of a specific albumin from its natural source, and the separation of other bodies, the following description of the preparation of a typical vegetable albumin, viz., **edestin,** may here be given.

A quantity, say 500 grams, of hemp-seed is ground up and the fat thoroughly extracted by shaking in a large flask with light petroleum and pouring off the solution. After draining off as much as possible of the petroleum, the remainder may be allowed to spontaneously evaporate. The residue is then digested at 60° C. with 350 c.c. of 5 per cent. salt solution, with continual stirring. The liquid is filtered through calico and allowed to cool. A precipitate forms, which can be washed by decantation with distilled water. It is redissolved in 250 c.c. of 5 per cent. salt solution, and the solution filtered through a warm filter. On cooling crystals of edestin separate, which can be washed successively with cold 5 per cent. salt solution, distilled water, alcohol and ether.

CLASSIFICATION OF ALBUMINS

We are now in a position better to appreciate the following classification of albumins and related substances. Where the name of the substance does not indicate its source or characteristic properties, short explanatory notes are added.

Group I

Albumins Proper.—These are naturally occurring substances and are all typical colloids :—

1. Serum albumin, lact-albumin, egg-albumin.
2. Serum globulin, lacto-globulin, cell globulin.
3. Plant globulins and vitellins.
4. Fibrinogen. (Occurs in the blood plasma of all vertebrates.)
5. Myosin and allied substances. (Derived from muscle.)
6. Phosphorus containing albumins, casein, vitellins and the nucleo albumins of the cell protoplasms.
7. Protamines. (These occur in the spermatozoa of fishes, etc.)
8. Histones. (These do not occur in the free state but in combination with other complexes, to form substances such as hæmoglobin.)

Group II

Disintegration Products of Group I :—

1. Acid albumins, alkali albumins.
2. Albumoses, peptones and peptides.
3. Halogen compounds of albumins, etc.

Group III

Proteids.—These are compounds of albumins with other complex groups which have been termed prosthetic groups :—

1. Nucleo proteids, compounds of albumin with nucleic acid.
2. Hæmoglobin and allied substances. (Hæmoglobin is

the red colouring matter of the blood, and consists of an albumin compound with a prosthetic group, which in this case gives rise to colouring matter and is therefore called a chromatogenic group.)

3. Glycoproteid sand mucins occurring in mucus. In this case the prosthetic group is a residue of a carbohydrate.

Group IV

Albuminoids.—These are many of them rather ill-defined bodies which form part of the skeletal structure of the animal or plant organism. The classification is mainly anatomical.

1. Collagin, gelatine. (The sub-stratum of bone and cartilage consists of collagin; on boiling with water it yields gelatine or glue.)
2. Keratin. (The chief constituents of the horny substances of mammals and birds.)
3. Elastin. (Occurs in certain fibrous animal tissue.)
4. Fibroin. (Occurs in raw silk.)
5. Spongin. (Forms the frame-work of the bath-sponge.)
6. Amyloid. (A pathological product, sometimes found in the brain, liver, etc.)
7. Albumoids. (Sundry substances found in various animals, membranes, etc., difficult to classify.)
8. Colouring matters derived from albumins, e.g., melanin, the pigment substance of the skin of dark-skinned races.

It may be useful shortly to summarise the information in the foregoing chapter as follows :—

Albumins or *Proteins* are complex nitrogenous colloidal substances occurring in animal and vegetable protoplasm, etc., and capable of being separated by their varying solubility in solutions of certain salts (pp. 204–207).

These yield, on treatment with *dilute* acids or alkalis, solutions containing *acid or alkali-albumins* (p. 185).

By heating with *acids* or by the action of *enzymes* such as *pepsin* or *trypsin*, albumins are gradually broken down, yielding successively :—

1. *Albumoses* precipitated by alcohol and by ammonium sulphate.

2. *Peptones* precipitated by alcohol but not by ammonium sulphate.

3. *Polypeptides*, compounds which still give the biuret reaction, are capable of synthesis by the condensation of amino-acids, and can be further broken down to (pp. 201–202)

4. *Amino-acids* and related substances, known as primary disintegration products (pp. 192–201).

BACTERIA AND PROTEOLYSIS

If an ordinary plate culture is made from a small quantity of sewage the gelatine will be found to liquefy round several of the colonies. This liquefying action is not infrequently so rapid and intense that a few liquefying organisms will cause the whole plate to become liquid, before the remaining colonies have time to develop.

Such organisms manifestly play an important part in the disintegration of albuminous matter.

The action of these and other bacteria on the organic matter of sewage has been the subject of a research by Messrs. Clark and Gage of the Massachusetts State Board of Health. They compared some 300 cultures of sewage bacteria, as regards their ability to produce ammonia in peptone solution, to reduce nitrates in nitrated peptone solution, and to liquefy organic matter in the form of gelatine, during an incubation period of seven days.

The peptone solution consisted of 0·1 per cent. Witte's peptone in distilled water, which gave an organic nitrogen

value (determined by the Kjeldahl method) of 14 parts nitrogen per 100,000. The nitrated peptone solution contained, in addition, nitrate equivalent to 10 parts nitrogen per 100,000. The liquefying power was determined by taking test-tubes of uniform bore filled to a depth of 100 mm. with standard beef peptone gelatine. The entire surface was inoculated, and the depth of liquefaction was measured after a given time.

The general result of these researches was to show that, as a rule, the liquefying power was synonymous with increased ability to reduce nitrates and to ammoniafy peptone.

In order to determine whether a liquefying organism secretes a proteolytic enzyme, about 0·5 per cent. of thymol may be added to the liquefied gelatine, to inhibit further bacterial activity, and a measured quantity of the liquid thus obtained, say 0·1 c.c., placed on the surface of nutrient gelatine, containing also 0·5 per cent. of thymol, in a tube of uniform bore. The liquefaction of the gelatine can be readily observed, and by taking different strengths of the liquid containing the enzyme, quantitative measurements can be made.

Reference may here be made to the activity of proteolytic organisms in the so-called ' bating ' or ' puering ' process in the **tannery.** In this process the skins, which have been ' dehaired ' by lime, are immersed in a bath or ' bate ' of pigeon's or dog's dung. The bacteria present produce digestive enzymes, which have a solvent action on the fibres of the skin, rendering it more supple. At the same time the acids, ammonia and amines which are produced assist in the solution of the lime remaining in the skin from the de-hairing operation.

In order to avoid the use of the unpleasant ' bate ' or ' puer ' above mentioned, and with the object also of more accurately controlling the process, J. T. Wood, in conjunction with Popp and Becker, has successfully made use of a puer-substitute, termed ' erodin,' which consists of a culture medium of peptonised gelatinous tissue, with a special mixed culture of selected bacteria.

CHAPTER XIII

THE NITROGEN CYCLE

We have seen in the chapter on the chemistry of albumins that substances comprised under this term constitute the basis of both animal and vegetable living matter. We know that the nitrogen in our food stuffs occurs mainly in the form of albumin, either animal or vegetable. The vegetarian, if he does not consume eggs, must at any rate add to his diet a considerable proportion of beans and peas, which are rich in vegetable albumin. The actual amount of nitrogenous food needed for useful work is a vexed question and need not here be considered, our object being confined to following out the chemical history of the nitrogen whether large or small in quantity. Used as food we have already learned that peptic and tryptic digestion of albumin leads by gradual stages to the formation of end products, largely consisting of amino acids. These one would not expect to be excreted as such from the body; they are built up again into the body substance through the biotic energy of the cells, and a portion also will be used up as fuel for maintaining that energy; consequently, therefore, we do not find in the products of excretion of the animal body just those amino acids and polypeptides which are formed when albumin is digested by pepsin or trypsin, under laboratory conditions.

Some of these substances, it is true, are found amongst the products of excretion; thus leucin and tyrosin have been

mentioned as occurring under certain conditions in human urine, and ornithin is so named from its occurrence in the urine of birds. Skatol and indol are characteristic constituents of fæces. In the case of flesh-eating mammals, however, by far the greater proportion of the nitrogen, which is not used up in adding to, or maintaining, the body substance, is excreted in the form of **urea** contained in the urine. Urea is a comparatively simple substance of which the chemical formula is $CO(NH_2)_2$; chemically it is known as carbamide, being the amide of carbonic acid, $CO(OH)_2$. The proportion of urea in the urine is, in fact, an index as to whether proper physiological equilibrium is being maintained, and its determination in the urine is a routine test in medicine. Its estimation depends on the fact that it is decomposed by sodium hypobromite, with liberation of nitrogen, according to the following equation :—

$$CO(NH_2)_2 + 3NaBrO = CO_2 + N_2 + 2H_2O + 3NaBr$$

Urea is also broken up in a similar manner by nitrous acid, obtained by adding a mixture of sodium nitrite and sulphuric acid to the solution containing the urea. In this case nitrogen is evolved both from the nitrous acid and the urea in equal proportion, according to the following equation :—

$$CO(NH_2)_2 + 2HNO_2 = CO_2 + 3H_2O + 2N_2$$

This reaction is of far-reaching importance, as it probably represents one method by which the nitrogen, originally consumed as albumin food, finally reappears as free nitrogen. In the case of animals whose diet is wholly vegetable the greater part of the nitrogen is excreted as so-called hippuric acid or benzoyl glycocoll, which has the formula

$$C_6H_5CONHCH_2COOH$$

We have now to consider how these two main end products of nitrogen metabolism, viz., urea and hippuric acid, are reabsorbed into the cycle of nature. They are not in themselves directly available for plant food, and the first stage in their reabsorption by plants, whose nitrogen may serve again as food for animals, consists in their conversion into ammonia. That the conversion of urea into ammonia was a fermentation process, and therefore due to life agency in some form, most probably to bacteria, was first suspected by Pasteur, and also by Tiegheim. Subsequent investigators showed that numerous organisms can induce ammoniacal fermentation. The most active of these is a micrococcus known as *Micrococcus ureæ*, and also a bacillus, *Bacillus ureæ*. These organisms are very widely distributed, and consequently urine, if left exposed to the air, very rapidly becomes ammoniacal, and the strong smell of an ill-kept urinal is thus accounted for. In normal health it has been shown that these organisms are not present in freshly excreted urine.

To demonstrate the ammoniacal fermentation of urea, some 50 c.c. of fresh urine may be taken and diluted with an equal volume of water in a conical flask, thus exposing a large surface to the air ; the solution may be infected with a drop or two of ammoniacal urine, or with a few centigrams of garden soil, and allowed to stand with occasional shaking for some days. A similar solution may be made up with similarly infected urine, and a small bottle completely filled with it, and stoppered. Both flask and bottle may be placed in the incubator at a temperature of 26° C. (80° F.) ; in a day or two both solutions, on testing with litmus paper, will be found to have become strongly alkaline, and Nessler reagent will reveal the presence of considerable quantities of ammonia. It is evident from this experiment that ammoniacal fermentation of urea can take place both under *anaerobic* and *aerobic* conditions ; the organisms of ammoniacal fermentation belong therefore to the class known as *facultative* aerobes.

This circumstance is of considerable importance in connection with the purification of sewage. If the fermentation is allowed to proceed till approximate completion, and a drop of the solution is examined under the high-power microscope ($\frac{1}{12}$ inch oil immersion), the micrococcus can be plainly seen.

Similar results are obtained if, instead of urine, an artificial solution is made up in the following proportions and similarly fermented :—

Water	1500 grams
Urea	33 ,,
Sodium chloride	18 ,,
Potassium hydrogen phosphate ..	5 ,,
Magnesium sulphate	0·5 gram

The reaction which takes place in both these cases consists in a simple hydrolytic change resulting in the formation of ammonium carbonate, thus :—

$$CO(NH_2)_2 + H_2O = (NH_4)_2CO_3$$

It has been found that the same organisms which bring about the conversion of urea into ammonia will also decompose uric acid, with production eventually of ammonia ; and hippuric acid, with formation of benzoic acid and glycin (glycocoll or amino-acetic acid), according to the following equation :—

$$C_6H_5CONHCH_2COOH + H_2O = C_6H_5COOH + CH_2NH_2COOH$$

Hippuric acid or benzoyl-glycin — Amino-acetic acid or glycin

Like all other fermentations, the ammoniacal fermentation ceases when a certain concentration is reached. In this case fermentation proceeds until the ammonium carbonate formed reaches a concentration of 13 per cent.

The ammoniacal fermentation belongs to an increasing number of such changes, which can ultimately be referred to the activity of a non-living enzyme. In 1874 Musculus found that if ammoniacal urine was filtered through filter paper, and the filter paper was washed and dried and afterwards placed in a neutral solution of urea, ammoniacal fermentation took place. This also happened if the filter paper was washed with strong alcohol, showing that the activity was due to something other than the living organism. Although not absolutely conclusive, the evidence at present available indicates that the micro-organisms secrete an enzyme which has been termed **urease**; it can be precipitated by alcohol and is destroyed by acids. Sheridan Lea in 1885 obtained a rapid ammoniacal fermentation of a 2 per cent. solution of urea, by incubating it at 38° C. with the alcoholic precipitate obtained from pathological urine. Sheridan Lea concluded that urease was soluble in water after the cells had been killed by alcohol, but that otherwise it was intracellular. It can hardly be said that Sheridan Lea's experiments are quite convincing; the writer has endeavoured to repeat them with ordinary urine, so far with little success. The existence of urease, apart from the organism, whether the latter is in a living state or in the form of its dead cells, is not, in the writer's opinion, as yet fully established, and it is possible, therefore, that the cell substance itself may not be without effect upon the reaction. Be this as it may, the essential fact remains that the nitrogen of albuminoid material appears in the course of the digestive process of animals, and of the putrefactive changes taking place in nature, in the form of amino acids or urea, which are apparently not available for plant food until they have undergone the ammoniacal fermentation which has just been described. Nitrogen in the form of carbonate of ammonia is capable of serving as plant food; in the plant it is built up again into vegetable albumins which form the food of animals.

Nitrification.—Not only can plants absorb their nitrogen in the form of ammonia, but they can also make use of products of oxidation of ammonia, viz., nitrites and nitrates. The chemical equations showing the relation between ammonia and nitrous and nitric acids are as follows :—

$$NH_3 + 2O_2 = HNO_2 + H_2O + O = HNO_3$$

It is possible in the laboratory directly to oxidise ammonia to nitrous acid by passing electric sparks through a mixture of ammonia and oxygen, or by passing the mixture over heated spongy platinum.

It was Pasteur who first suggested that the oxidation of ammonia to nitric acid, which evidently takes place in nature, was really due to micro-organisms, and two French chemists, Schlösing and Muntz, actually proved that this was the case. They found that if solutions containing ammonia were allowed to percolate through soil, which was well aerated at regular intervals, the ammonia was mainly converted into nitrate ; but that if any living energy in the soil was paralysed, e.g. by the introduction of chloroform vapour, or by other antiseptics, no nitrification took place. The study of the conditions of nitrification has engaged the attention of a great number of workers both in England and on the Continent, and is of the very greatest importance from the point of view of agriculture, and the kindred subject of sewage purification. In order to have a living idea of the sequence of changes which take place when the nitrogenous solution undergoes nitrification, the following experiment may be undertaken: 10 c.c. of urine may be added to a litre of water in a Winchester bottle, together with about a gram of good garden mould, and the solution, which will occupy rather less than half of the bottle, may be continually aerated, either by drawing air through by means of a Bunsen water pump, or by attaching the bottle to a

shaking machine. At intervals of about three days about 20 c.c. of the solution may be examined :—

(*a*) for ammonia by means of Nessler solution ;

(*b*) for nitrites by means of acetic acid, potassium iodide and starch, and

(*c*) for nitrates by means of the Stoddart test.

The following further details in regard to these tests may be useful :—

Ammonia gives a reddish-brown precipitate, or in dilute solutions, a yellowish-brown coloration, with an alkaline solution of potassium mercury iodide, known as *Nessler's reagent.* The depth of coloration is proportional to the amount of ammonia present.

Nitrites.—Acetic acid liberates nitrous acid from a solution containing nitrites ; the nitrous acid, in its turn, liberates iodine from potassium iodide, and the free iodine gives a blue coloration with starch.

Nitrates.—The Stoddart test affords a ready means of determining the presence of nitrate : 10 c.c. of the sample, filtered from suspended solids, are poured into a test-tube of rather thick glass. About as much pyrogallol as will cover a sixpence is then dissolved in the solution and 2 c.c. of strong nitrate-free sulphuric acid carefully added from a pipette, so as to form a layer in the lower portion of the solution. Dry powdered sodium chloride (salt) about equal in quantity to the pyrogallol is now added, and if nitrate is present a purple band is formed immediately above the sulphuric acid layer. The intensity of the coloration is roughly proportional to the amount of nitrate present.

It will be found that a progressive change takes place ; first of all, formation of ammonia will be noticed, with no nitrite or nitrate ; this attains a maximum, and then decreases with simultaneous appearance of nitrite, but little or no nitrate ; finally the nitrites disappear and there is left a

solution containing only nitrate. During the course of the experiment the bottle should be kept as far as possible in darkness, to prevent the formation of green algæ growths, which combine with the nitrogen of the ammonia or the nitrate, and so confuse the progress of the reaction.

Experiments of this kind were carried out by Munro in 1883, who showed that practically every form of nitrogenous organic matter was capable of undergoing this series of changes.

We are indebted to the labours of Warington for the exhaustive study of the conditions under which nitrification occurs.

He showed that the power of nitrification could be communicated to solutions, which otherwise did not nitrify, by inoculating them from solutions in which nitrification was taking place.

He further confirmed the results of Schlösing and Muntz by showing that nitrification could be inhibited by the introduction of antiseptics such as chloroform and carbon bisulphide.

The following were the conditions which Warington found to be essential for nitrification, and his results are in harmony with those of other observers, among whom may be especially mentioned Munro and Wienogradski.

1. It was found that phosphates are the essential element of the food of the organism of nitrification. In fact, the very interesting observation was made that these organisms could thrive on purely inorganic material, and even that the presence of organic matter appears to have an inhibiting effect. This question will be further considered in the light of more recent investigations.

2. The presence of oxygen is essential to the activity of the nitrifying organisms.

3. The presence of a base is also essential to neutralise the nitrous and nitric acids as they are formed ; at the same time there must not be an excessive alkalinity.

4. Like other organisms the nitrifying organisms have an optimum temperature of activity; they will produce effects at as low a temperature as 3° or 4° C. (37° or 39° F.), they are fairly active at 12° C. (54° F.), but they work best at 37° C. (99° F.). Still higher temperatures begin to be prejudicial, and like other organisms they are apt to be destroyed by strong sunlight. The latter circumstances, it may be mentioned, are believed by Major Clemesha to account for the absence of nitrates in certain surface waters in India.

These facts have a very important bearing on the processes of agriculture and especially also those of sewage purification. The experiment which is described on p. 217 indicates clearly that nitrification proceeds in two stages, the ammonia being first oxidised to nitrite and then to nitrate; it has been found that these two reactions are the work of separate organisms.

Warington was not successful in isolating either of these, partly for the reason that neither organism will grow on gelatine. Wienogradski in Russia, and Percy Frankland in this country, independently made use of gelatinous silica as a means of cultivation. The solution used with which the silica was gelatinised had the following composition in the case of the nitrous organism :—

2 grams ammonium sulphate;
0·5 gram magnesium sulphate;
2 grams sodium chloride;
0·4 gram ferrous sulphate;
1000 c.c. of water.

The nitric organism is more difficult to isolate even than the nitrous, as it is much smaller. Wienogradski, however, succeeded in 1891; he made use of the following solution :—

1 gram potassium hydrogen phosphate ;
½ gram magnesium sulphate ;
trace of calcium chloride ;
2 grams sodium chloride ;
1000 c.c. water.

Twenty c.c. of this solution were placed in a flat-bottomed flask and a little freshly washed magnesium carbonate added, the flask was closed with cotton wool and sterilised ; 2 c.c. of a 2 per cent. solution of ammonium sulphate were then added and the whole inoculated with a little soil. When nitrate development had taken place subcultures were made on to silica jelly. The researches of Frankland and Wienogradski have been confirmed by other investigators.

From the detailed work of Boullanger and Massol, it appears that there are two well-defined organisms which convert ammonia into nitrites. *Nitrosomonas*, which is a fairly large, nearly spherical organism, exists in two varieties, one the form usually found in Europe, and the other in certain soils occurring in Java. There is also a smaller form known as *nitrosococcus*.

The *nitric* organism is a very small bacterium whose length somewhat exceeds its breadth.

These two organisms, the nitrous and the nitric, work together in nature, and neither can do its work without the help of the other ; the nitric organism is incapable of directly oxidising ammonia, and the nitrous organism cannot carry the oxidation of ammonia farther than the stage of nitrite. A very important consequence of this differential action is seen in the changes which take place when sewage matter is discharged into sea water ; the nitrifying organism under these conditions is either actually destroyed or rendered inactive. Dr. W. E. Adeney gives the following figures for the results of spontaneous oxidation of sewage, and comparative mixtures of sewage and fresh water, and sewage and sea water, respectively :—

—	Parts per 100,000		
	Sewage	Sea Water Mixture	Fresh Water Mixture
At commencement—			
Nitrogen as ammonia ..	0·825	0·165	0·165
Nitrogen as Nitrites ..	0·0	0·00	0·00
Nitrogen as Nitrates ..	0·0	0·01	0·01
Organic Nitrogen	0·675	0·135	0·135
At conclusion—			
Nitrogen as ammonia ..	0·02	0·0	0·0
Nitrogen as nitrites ..	0·0	0·14	0·0
Nitrogen as nitrates ..	0·92	0·0	0·142
Organic nitrogen	0·5	0·072	0·076

The author has confirmed these observations in experiments made for the purpose of tracing the changes taking place when sewage sludge is discharged into sea water; he found, not only that the ultimate product of oxidation of nitrogen was nitrite rather than nitrate, but also that the actual oxidation of ammonia took place more slowly in sea water than in fresh water.

He has also noticed the same phenomenon of the production of nitrite, rather than of nitrate, in a case where sewage effluent was being discharged into a stream containing large quantities of calcium chloride from an ammonia soda works.

So far we have considered, primarily, the oxidation of solutions containing ammonium salts, with no admixture of organic matter, and with more or less pure cultivations of the nitrous and nitric organisms. In nature, however, such conditions of course do not obtain; we have there to do with organic matter in different stages of decomposition, and with

mixtures of numerous organisms. The conditions, under which the final nitrification then takes place, have been worked out by Adeney in a series of very careful researches. His method of research consisted in exposing solutions, either of defined chemical substances such as urea, asparagin, ammonium tartrate, etc., or less defined organic matter such as town sewage, or infusions of peat, to the prolonged action of oxygen, in the presence of the usual organisms to be found in natural waters. This was accomplished either by mixing the solution with a known volume of aerated tap water, or by shaking the solution periodically with known volumes of air. Not only were the products of decomposition and oxidation determined, such as ammonia and nitrous and nitric acid, but also the carbonic acid resulting from the oxidation of the carbonaceous matter present, as well as the resulting change in composition of the dissolved gases present. For this purpose Adeney devised a special form of gas analysis apparatus, which enabled him to analyse the gases obtained on boiling out the solutions *in vacuo*. He discovered the source of error in previous determinations, viz., the fact that the carbon dioxide formed by oxidation of organic matter is present largely as carbonate, and is only fully recovered from the solution if the latter is acidified before boiling. The oversight of this fact led Sir Edward Frankland to conclude that the rate of oxidation, e.g. of sewage matter, when discharged into a stream, was much less than was actually the case. As a result of prolonged investigation, Adeney arrived at the following conclusions :—

Oxidation of organic matter proceeds in two well-defined stages, which may be briefly described as the *carbon* oxidation stage, and the *nitrogen* oxidation stage.

In the carbon oxidation stage, carbon dioxide, water, ammonia, and excretory substances are produced ; in the second or nitrogen oxidation stage, the two last-named bodies are further fermented, the products being nitrites, nitrates,

and comparatively small quantities of carbon dioxide. He confirms the conclusions of previous observers, by showing that in solutions of organic matter the nitrous organisms thrive, while the nitric organisms lose their vitality. He also finds that the nitrous organism cannot carry oxidation beyond the stage of nitrite, whereas the nitric organism only oxidises nitrites to nitrates. He adds the further important conclusion, that the presence of peaty or humus matter appears to preserve the vitality of the nitric organisms, during the earlier stages of the fermentation process, and establishes conditions whereby it is possible for the nitric organisms to thrive simultaneously with the nitrous. This latter conclusion has an important bearing on the oxidation of organic matter in nature, and especially under the controlled conditions which obtain in modern processes for the biological purification of sewage.

In all the researches on the nitrifying organisms referred to in the foregoing pages, the conditions have been essentially laboratory conditions, where the solutions of organic matter have been exposed to air, so to speak, in bulk, either by simple exposure of a solution in a flask, by shaking with air, or by bubbling air through ; the element of surface action has not been brought into play. It is clear on reflection that if the solution to be nitrified could be passed in a thin film over a large surface, with free circulation of air, the conditions for oxidation would be very much more favourable ; for not only would the presence of ample oxygen be assured, but also the extended surface would afford a substratum for a greatly increased development of the necessary organisms. It is the application of these principles which has led to the modern developments in sewage purification processes.

In 1869 Sir Edward Frankland, acting on behalf of the Royal Commission on Sewage Disposal then sitting, made his classical experiments on the so-called intermittent filtration of sewage through soil. He made use of cylinders fifteen feet

in height filled with sand or earth, and dosed them with defined quantities of sewage, allowing intervals for aeration between each dose. By this method he was able to purify much greater quantities of sewage on a given surface area of soil, than by the so-called broad irrigation processes formerly in vogue. At that time, however, the true explanation of the oxidation change which took place was not properly understood, and it was considered to be a purely chemical phenomenon. Later on the Massachusetts State Board of Health took up the subject, in the light of the researches of Warington, Wienogradski, and Percy Frankland, and they worked out the conditions for the successful oxidation of sewage matter by percolation through sand filters. They showed that the results depended essentially upon the presence of oxygen, and upon the time allowed for the change to take place. They confirmed Warington's conclusion that it was necessary for a base of some kind to be present, to combine with the nitrous and nitric acid produced by the oxidation of ammonia ; all other conditions they considered were secondary to these three.

It was Stoddart who showed in 1893 that the time factor could be gradually decreased, if filters of more open material than sand were used, and care was taken to distribute the nitrifying solution in such a way that a thin film only was exposed to the action of the air. By allowing a solution of ammonium carbonate (1 part N in 10,000) to drip on to a column of coarsely powdered chalk properly inoculated with nitrifying organisms he was able to obtain highly efficient nitrification.

This experiment of Stoddart's is really the original of the modern trickling or percolating sewage filter.

Scott-Moncrieff in 1898, by employing superimposed trays of filtering medium for the final purification of sewage, which had undergone preliminary ammoniacal fermentation in a so-called 'cultivation tank,' obtained a high degree

of nitrification. A very interesting result was also demonstrated by these experiments, viz., that the nitrification was a progressive phenomenon, and its course was considerably interfered with if, after it had once been established, the sequence of the trays was altered, the last tray, e.g., being substituted for the highest, in which case the nitrification was considerably impeded, until the original conditions were re-established.

The bacteriological conditions obtaining in sewage filters of this description have been worked out in recent years by Boullanger and Massol at the Pasteur Institut at Lille, by Schulze-Schulzenstein in Germany, and by Dr. Harriette Chick of the Lister Institute. All these investigators agree that the nitrifying organisms found in ordinary sewage filters are the same as those which occur in soil. Boullanger and Massol have found an explanation for the seeming discrepancy between the results of Wienogradski and those which are obtained on sewage filters. According to Wienogradski, it will be remembered, the activity of the nitrifying organism is inhibited by the presence of ammonia or of organic matter. Boullanger and Massol concluded from their experiments, that while the presence of large quantities of ammonia or of organic matter may impede the original development of the nitric organism, yet if the growth of this is once established, its activity is unaffected by these conditions. These results are in harmony with Adeney's conclusion that the presence of peaty matter is of assistance in maintaining the activity of the nitric organism. In a sewage filter the extended surface enables an abundant growth of nitrifying organism to take place; at the same time it is well known that if the maximum load, as it were, of sewage matter is put upon the filter in its early stages, before nitrification is established, it is difficult, if not impossible, for the right conditions to be set up later. It is consequently necessary to 'ripen' the filter, by putting on only comparatively small quantities

of sewage at first, increasing the quantity as nitrification becomes established.

Dr. Chick found that in sewage filters, as in the experiments with solutions, the nitrification took place in two well-defined stages, first nitrites and then nitrates being formed. The length of time required for complete nitrification to become established depended on the amount of ammonia present, either actually as ammonium carbonate, or potentially as unfermented organic matter in the sewage applied. Temperature also has a marked effect, as might be expected, in determining the time necessary for nitrification to be established; for this reason it is advisable always if possible to bring new sewage filters into work during the warmer months of the year.

Finally, mention may be made of the importance of the character of the material used in the construction of the filter beds. Practical experience has shown that better results are obtained with a medium which offers a maximum of surface; thus irregular material, such as clinkers, gives better results than when a smoother material, such as gravel, is used. Experiments by the author and Percy Gaunt have shown that, in addition to the effect of surface in giving an extended habitat for bacteria, the majority of vesicular or porous materials have the power, to a greater or less extent, of retaining ammonium salts, either in their smaller pores or in their larger interstices; such materials, therefore, afford a somewhat longer time for the nitrifying action to take place when a solution containing ammonium salts is brought in contact with them.

The purely physical side of this question has also been carefully investigated by W. Clifford. He allowed known amounts of water to trickle through media of different kinds and dimensions at defined rates. When equilibrium was established between the rate of inflow and outflow, a known amount of sodium chloride solution of known strength was run on to the filter. The amount of chlorine emerging from the

medium was determined at defined intervals. He afterwards allowed the medium to drain and measured the amount of drainage water, and finally dried the medium, and determined the loss of moisture. He thus measured for each class of medium (*a*) the amount of water passing through in a given time, (*b*) the amount of water held in the larger interstices, and (*c*) the amount of water retained in the pores. These experiments showed generally that the time of percolation through clean filter material varies, inversely as the rate of application of the water, and directly as the amount of water taking part in the water movement through the bed. This latter obviously depends on the size of particles, and the physical character of the medium.

These results find expression in the following formula :—

$$c = \frac{I}{RT}$$

where c is a constant, I the interstitial water per cubic yard, R the rate of sprinkling per square yard per hour, and T the average time of sprinkling through three feet of medium.

Unpublished experiments by the author and Mr. T. W. Lockett have shown that when a nitrifying solution, made up after Wienogradski's recipe, is allowed to drip on laboratory filters, composed respectively of quartz particles about $\frac{1}{8}$ inch diameter, and of broken clinker of the same dimensions, nitrification is established much more rapidly in the case of the clinker medium than in the case of the quartz.

De-nitrification.—De-nitrification, as the name implies, is the reverse of nitrification. De-nitrification changes are concerned either with :—

(1) The reduction of nitrates to nitrites, or ammonia ; (2) the reduction of nitrates and nitrites to oxides of nitrogen,

NO and N_2O; or (3) the reduction of nitrates and nitrites to nitrogen.

The first characteristic work on this subject was done by Gayon and Dupetit in 1882. They found that when a solution containing potassium nitrate, together with sewage and a little urine, was allowed to stand in absence of air, the nitrate was reduced. When using nitrated broth containing *asparagin*, they obtained an evolution of nitric oxide; they also noted the effect on the reaction due to the addition of carbohydrates and tartrates, etc., and they concluded that de-nitrification was essentially the combustion of organic matter by the oxygen of the nitrates. It thus naturally proceeded best in presence of a minimum air supply. It could be shown, e.g., that in a given solution a greater amount of de-nitrification took place in the lower portion of the solution than at the surface.

The subject of de-nitrification has been investigated by numerous workers, notably Percy Frankland and Beyerinck.

The latter describes an elegant experiment for the demonstration of the presence of de-nitrifying organisms in sewage. 0·1 per cent. of potassium nitrate and a little starch paste is added to nutrient gelatine, and the whole sterilised and poured into a Petri dish. A little sewage, diluted, say twenty times, with distilled water is poured on and off the plate, which is turned with the gelatine surface downwards and allowed to grow at 20° C. When the colonies have developed, a dilute solution of hydrochloric acid and potassium iodide is poured over half the plate. Wherever nitrites have been formed, iodine will be liberated and will colour the starch blue. Colonies on the other half of the plate, similar in appearance to those giving the blue starch-iodide reaction, and which will not have been killed by the acid, may be picked out and grown separately in suitable solutions.

For the study of the ultimate conversion of nitrate into nitrogen, the following solution may be made use of:—

1 litre of river water;
2 grams calcium tartrate;
0·5 gram potassium hydrogen phosphate;
0·1 gram potassium nitrate.

This is sown with a little horse dung, or straw, and incubated at 35° C. The general reaction taking place may be expressed by the following equation:—

$$4KNO_3 + 5C + 2H_2O = 4KHCO_3 + 2N_2 + CO_2$$

It will be remembered that when the decomposition of cellulose under aerobic conditions was being considered, a mixture was made of a similar character to the solution just described, the carbon being represented by the carbon of cellulose; the importance of de-nitrification as a natural phenomenon is thus seen. On the one hand, we have the nitrifying organisms oxidising ammonia to nitrite and nitrate, while on the other hand the de-nitrifying organisms make use of the nitrate thus formed, to oxidise organic matter.

De-nitrification is by no means so restricted a phenomenon as nitrification, and quite a large number of organisms have been found which are capable of bringing about de-nitrification to a greater or less degree. Broadly speaking, these may be classified into two classes, true de-nitrifying organisms which are capable of pushing the reaction to its final limit and producing free nitrogen; and indirect de-nitrifying organisms, which only reduce nitrates to nitrites, when, through the interaction of nitrites with amido compounds in acid solution, as in the case of urea, we have:—

$$\underset{\text{Urea}}{CO(NH_2)_2} + \underset{\text{Nitrous acid}}{2HNO_2} = 2N_2 + CO_2 + 3H_2O$$

Or to take an analogous, but more complicated, instance, asparagin may be converted into malic acid, thus:—

$$\begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{CHNH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CONH_2} \\ \text{Asparagin} \end{array} + 2\mathrm{HNO_2} = \begin{array}{l} \mathrm{COOH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CHOH} \\ | \\ \mathrm{COOH} \\ \text{Malic acid} \end{array} + 2\mathrm{N_2} + 2\mathrm{H_2O}$$

Recently a somewhat sensational discovery has been made by Beyerinck and Minkman. Besides identifying the de-nitrifying organism originally discovered by Gayon and Dupetit, they have isolated two other organisms, which are probably the destroyers of nitrates in the soil. They describe the following experiment:—

A bottle, with a well-fitting glass stopper, is filled with bouillon, containing 8 per cent. of potassium nitrate, and 10 to 20 grams of garden soil are added. After incubation at 37° C. for a day or two, a considerable froth forms, which forces out the liquid by a capillary action between the stopper and the neck of the bottle. The gas evolved remains in the bottle, and on cautiously opening the bottle at the end of forty-eight hours, and applying a glowing chip, it will burst into flame through the action of the nitrous oxide present. This has been found to amount to as much as 90 per cent. of the gases evolved. They have also isolated a second organism, which is capable of causing the combination of hydrogen and nitrous oxide, when these two gases are simultaneously led into the solution containing the organism. From this combination the organism appears to derive energy which enables it actually to decompose carbon dioxide, and thus utilise the carbon for building up its own structure. This is an extremely interesting instance of the reabsorption of carbon from its final state of oxidation as carbon dioxide, back into the cycle of organic life. We know,

of course, that plants possess this property through the activity of the chlorophyll in their cells, but instances of the utilisation of the carbon in carbon dioxide by lower organisms have not been frequently observed.

Assimilation of Nitrogen.—It will be seen from the above equations, representing de-nitrification changes, that these must eventuate in escape of nitrogen into the atmosphere. If this continued, it is evident that in time the stock of nitrogen available for life would become depleted, as a certain percentage of the nitrogen of all organic matter would be permanently lost in this way. Fortunately a means exists for bringing back this escaped nitrogen once more into the cycle of life. A certain small quantity is returned as nitric acid, through the combination of nitrogen and oxygen brought about by the electric discharge of the lightning; and of recent years considerable developments have taken place in the production of nitric acid by the union of the nitrogen and oxygen of the atmosphere, by means of powerful electric discharges artificially produced. Nitrogen has also been recovered artificially from the atmosphere by the production of calcium cyanamide in the electric furnace, by heating mixtures of lime or chalk with charcoal at a temperature of 2000° C. in a current of air.

Calcium carbide is first formed, which combines with nitrogen to form calcium cyanamide, thus :—

$$CaC_2 + 2N = Ca\,CN_2 + C$$

Calcium cyanamide can be used as a source of nitrogen in agriculture, as it decomposes readily in presence of moisture, yielding calcium carbonate and ammonia, thus :—

$$CaCN_2 + 3H_2O = CaCO_3 + 2NH_3$$

All these artificial methods are dependent upon cheap electricity for their economic development, and the works

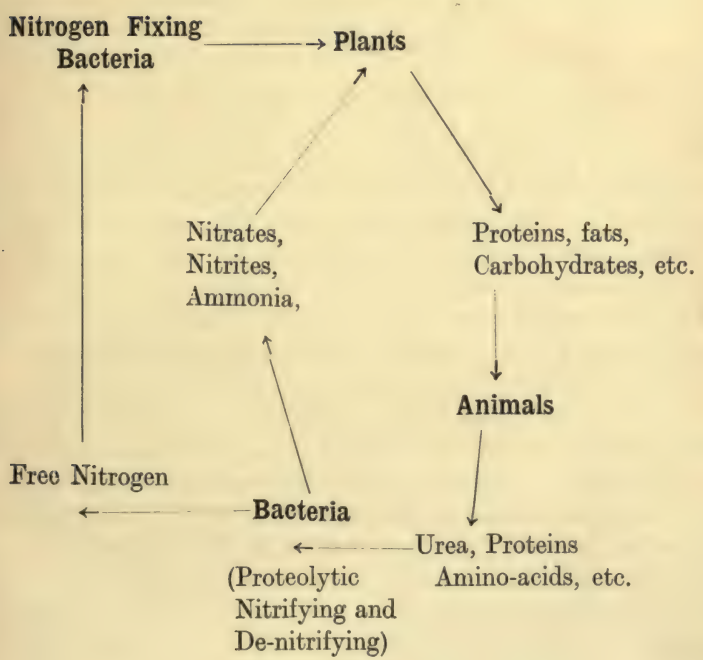
for their production are therefore situated mainly in Scandinavia, or in mountainous districts where water power can be readily utilised. The amount of nitrogen recovered by these artificial processes is, in the aggregate, of small account, compared with the silent but widely active processes of nature. The discovery of the natural process by which the apparent loss of nitrogen is made good is due to the researches of two German investigators, Hellriegel and Wilfarth. It will be of interest at this point to follow their discovery to some extent by making certain actual observations, if the season of the year permits.

If a fairly well-grown plant belonging to the Leguminaceæ, e.g., an ordinary garden sweet pea, be carefully pulled up by the roots and the latter examined, if necessary with a pocket lens, a number of little nodules will be observed on the rootlets (see Plate II (i)), which on pressing will exude a milky juice. If a microscopic preparation is made of this juice and it is examined under a high-power microscope, numerous bacteria will be found to be present. Hellriegel and Wilfarth found that plants, such as the sweet pea, were capable of growing in a sterile soil free from nitrogen, if this soil were treated with an extract of earth in which plants of the same family had been previously grown. The addition of this extract determined the development of the root nodules. They concluded that the nodule bacteria in some way assisted the plant to absorb its nitrogen from the air. Their conclusions were confirmed by Bréal, who compared the growth of rootlets of lupin, inoculated directly with the liquid contents of a root-nodule, with the development of similar rootlets which had not been inoculated. The growth, and the percentage of nitrogen in the resulting plant, was much greater in the former than in the latter case. It is common knowledge that peas and beans are the chief sources of nitrogen in a vegetable diet; we thus see how by the action of these organisms, in assisting plants of this character

to assimilate the nitrogen of the air, the nitrogen cycle is completed. The plants, or their seeds, furnish food for animals and men, which nitrogenous food, as we have seen, is broken down, first by the digestive processes of the body, and afterwards by micro-organisms, producing first ammonia, and finally nitrates, to serve once more as food for plants. It must be remembered, besides, that apart from the leguminous plants used in this way for food, a large proportion of the total growth of the plants of this order must suffer decay, and their nitrogen be returned directly to the soil. Indeed this method of returning nitrogen to the soil constitutes one of the ordinary processes of agriculture, and is part of what is known as the rotation of crops. After a crop has been grown, such as wheat, which tends to exhaust the soil of its nitrogen, it is customary to grow a crop of clover, which is afterwards ploughed into the soil. The clover in its growth absorbs large quantities of nitrogen from the air ; when it is ploughed into the soil it rots, and once more, through the changes which have been described, this nitrogen is converted into nitrate, which will again serve as food for wheat.

The series of changes which has been discussed in the foregoing chapters may be usefully summarised in the following diagram, which is self-explanatory. The application of the knowledge, summarised in this diagram, to the practical problems of agriculture and sewage disposal will be more fully discussed in Chapters XVI and XVII.

The Nitrogen Cycle



CHAPTER XIV

THE SULPHUR CYCLE

EVERYONE who has been confronted with a bad egg is aware of the unpleasant character of the final decomposition products of albumin. The product most easily recognised chemically is sulphuretted hydrogen or hydrogen sulphide, H_2S, whose presence is easily demonstrated by holding a paper soaked in a solution of lead acetate in its vicinity. The smell of a rotten egg is mainly due to this gas. Hydrogen sulphide is therefore often described as having a smell like rotten eggs.

It has been shown in Chapter XII that most varieties of albumin contain sulphur in greater or less proportion, and they are capable, like egg-albumin, when undergoing putrefaction, of liberating this sulphur as hydrogen sulphide. It is easily seen, therefore, that decomposing albuminous matter is capable of causing considerable nuisance from this source.

Sulphur appears to be an essential constituent of both animal and vegetable life, and a knowledge of its transformations as it passes from one to the other is of the greatest importance, especially in view of the possibility of nuisance being produced during the process.

The transformations which sulphur compounds undergo bear a rough analogy to the transformations of nitrogen considered in Chapter XIII. Just as the plant takes up nitrate to furnish the nitrogen for vegetable albumin, which nitrogen ultimately reappears, after passage through the animal organism, as urea and ammonia, to be finally again oxidised

to nitrates, so the sulphur supplied to the plant as sulphates becomes part of vegetable and animal albumins, which again break down, yielding hydrogen sulphide, and the latter is oxidised, either chemically or biologically, back to sulphate.

Moreover, just as nitrates are capable of reduction to form nitrites, and finally ammonia, so sulphates are capable of reduction to hydrogen sulphide.

In the sulphur cycle purely chemical reactions play a greater part than is apparently the case with nitrogen, but in all cases the sulphur transformations are capable of being facilitated by the activities of various organisms.

The chief workers on this important question of the natural sequence of combinations entered into by sulphur, have been Wienogradski in Russia, who has investigated the conditions under which sulphur is oxidised by certain specific sulphur organisms; Beyerinck and Van Delden in Holland, who have studied particularly the reduction of sulphates; and Letts in Belfast, who, while repeating Beyerinck and Van Delden's experiments, has, in conjunction with several of his students, made important original observations upon the conditions under which sulphuretted hydrogen is evolved, in the actual circumstances of certain estuaries.

It will perhaps be simplest to consider the subject under two heads :—

I. The production of hydrogen sulphide.
II. The oxidation of hydrogen sulphide.

I. Hydrogen sulphide can arise under natural conditions from the following sources :—

(*a*) The decomposition of albumin, as already stated ;

(*b*) The reduction of sulphates.

Both these changes are due to the action of various organisms.

Letts and McKay have also shown that carbon dioxide, itself produced by the decomposition of organic matter, can decompose sulphides, such as ferrous sulphide, FeS, yielding H_2S. Such sulphides can also be decomposed by fatty acids produced by other fermentations. The two sources, *a* and *b*, of sulphuretted hydrogen may now be separately considered :—

(*a*) *The decomposition of albumin.*—The formation of hydrogen sulphide by the decomposition of albumin, through the action of bacteria, can be readily demonstrated. If a few drops of lead acetate solution are added to a small bottle full of sewage, the bottle closed, and placed in an incubator for a day or two, the solution turns black from the presence of lead sulphide. The actual organisms capable of decomposing albumin, with formation of hydrogen sulphide, can be recognised by an elegant method suggested by Beyerinck :—

To ordinary nutrient gelatine, sufficient white lead is added to obtain a perfectly white plate ; when the medium is poured into the Petri dish, a little sewage diluted with distilled water is poured over the plate. After it is set, and as the colonies develop, black dots of lead sulphide will indicate the presence of these organisms, which are capable of breaking down albumin with production of hydrogen sulphide.

A very serious case of nuisance has for a long time existed on the shores of Belfast Lough. Here great quantities of a seaweed, *Ulva latissima*, flourish. Professor Letts has shown that this seaweed contains an abnormally high albumin content ; when deprived of its natural conditions of growth, the Ulva is capable of fermentation, apparently in two distinct and successive stages. The first stage results in the production of fatty acids, mainly propionic, together with carbon dioxide and hydrogen ; in the second stage of fermentation, in which a different species of micro-organism is concerned, sulphuretted hydrogen is produced.

It is not at present certain what are the exact sources, in the first place of the fatty acids, and in the second place of the

sulphuretted hydrogen. They may both be due to decomposition of the albumin of the weed, or on the other hand sulphides may be produced by reduction of the sulphates in the sea water, or in the tissues of the Ulva, and these sulphides are then decomposed by the fatty acids produced in the first fermentation.

The evidence points to the hydrogen sulphide being derived from the reduction of sulphates, rather than from the decomposition of the albumin, inasmuch as when comparative tests were made, by fermenting the Ulva in sea water, and tap water, respectively, sulphuretted hydrogen was much more readily evolved from the sea water experiment than from the tap water. The reduction of sulphates is clearly, then, a very important source of hydrogen sulphide.

Before considering this process in detail, however, it should be stated that the objectionable odour evolved, when organic matter is allowed to putrefy, is not solely due to hydrogen sulphide. Under certain conditions, very evil-smelling gases are evolved in which no trace of hydrogen sulphide can be discovered. These are probably organic sulphur compounds, such as mercaptan (C_2H_5SH), also amines, and substances such as skatol, etc., which are also products of albumin decomposition. It has been further found that the yield of sulphuretted hydrogen can be increased in many cases if a small quantity of flowers of sulphur is added to the fermenting mixture.

(*b*) *Sulphate reduction.*—As already stated, this change has been studied by Beyerinck and Van Delden. Beyerinck inoculated suitable solutions containing sulphates with small quantities of mud from the canals of Delft, and found that the best conditions for sulphate reduction were as follows :—

(1) No oxygen must be present.

(2) No acid formation must take place, and consequently little or no sugar should be present in the culture media.

(3) Phosphates and other suitable solids must be present.

(4) Nitrogen compounds are only required in very small quantities ; sufficient indeed is contained in ordinary tap water.

(5) The most favourable temperature for sulphate reduction is about 25° C. Beyerinck succeeded in isolating an organism which he termed *Spirillum desulphuricans* ; it is a strictly anaerobic organism, and this circumstance, in conjunction with its small need for nitrogenous nutriment, enables it best to grow in solutions which have been worked over by other organisms. These facts are of not a little practical interest. Those who have had to deal with samples of sewage and effluents will have noticed that such samples, if kept in stoppered bottles, may become in time practically clear, having only a small black sediment at the bottom ; but if they have been tightly stoppered, they may also retain considerable quantities of hydrogen sulphide. If this is removed by boiling, very little residual organic matter will be found to be present.

Stagnant polluted waters, e.g., the Manchester Ship Canal, show the same phenomenon. It is evident, in both these cases, that the nitrogenous organic matter is broken down by ordinary putrefactive organisms, and that final sulphate reduction takes place. In such cases sulphides, or hydrogen sulphide, will be found to constitute almost all the oxidisable matter left.

To demonstrate the reduction of sulphates, the following solution was made use of by Van Delden :—

Tap water	1000	grams
Common salt	30	,,
Sodium lactate	10	,,
Crystallised magnesium sulphate ..	8	,,
Potassium phosphate	0·5	gram
Asparagin	0·5	,,

This solution may be inoculated with a little sewage sludge, from which sulphate reducing organisms are seldom, if ever, absent.

Van Delden isolated an organism causing the reduction of sulphates in sea water, and found that it closely resembled *Spirillum desulphuricans*; he named it *Microspira estuarii*. Both these organisms, although as above stated they do not need large quantities of nitrogen, are not inhibited in their growth by organic matter, if they are present in pure culture. Under natural conditions the presence of organic matter facilitates the growth of other organisms, to the detriment of the sulphate reducing spirillae.

The reduction of sulphates is of special importance in relation to the discharge of sewage into sea water. There is no doubt that, in absence of sufficient dilution, putrefaction may set in, resulting, in the case of sea water, in sulphuretted hydrogen production; so that the nuisance may be much greater in the case of discharges into sea water than into fresh water.

Sulphate reduction has been compared to de-nitrification; it will be remembered that in the case of the reduction of nitrates the oxygen of the nitrate with the assistance of the de-nitrifying organism combined with the organic matter present. A similar reaction appears to take place in the case of sulphate reduction; thus in the above described solution, where the chief source at any rate of oxidisable material is sodium lactate, Van Delden suggests the following equation :—

$$2C_3H_5O_3Na + 3MgSO_4 = 3MgCO_3 + Na_2CO_3 + 2CO_2 + 2H_2O + 3H_2S$$

Experimental evidence supports the above equation fairly well.

II. Oxidation of Sulphur.—Unlike ammonia, whose direct oxidation by purely chemical means has been shown to take place to only a limited extent in nature, hydrogen sulphide readily oxidises in a variety of ways. The simplest is the direct oxidation to water and sulphur according to the simple equation :—

$$H_2S + O = H_2O + S$$

This change is hastened by the presence of certain metallic oxides, particularly those of iron and manganese; thus in presence of oxide of iron the following changes may take place :—

$$3H_2S + Fe_2O_3 = 2FeS + 3H_2O + S$$

In presence of oxygen and moisture FeS may readily oxidise to ferrous sulphate, $FeSO_4$, thus :—

$$FeS + 2O_2 = FeSO_4$$

And this may further oxidise with formation of ferric sulphate, thus :—

$$(FeSO_4)_3 + O + H_2O = Fe_2(SO_4)_3 + Fe(OH)_2$$

It is quite possible that pyrites, especially when found in coal, may owe its origin to the interaction of oxide of iron and the sulphides produced by the decay of vegetable matter. When such 'coal brasses,' as this form of pyrites is termed, is exposed to the air, it oxidises with formation of ferrous sulphate, or eventually, it may be, of ferric sulphate.

When black sewage mud is exposed to the air it turns brown and becomes acid, owing to the formation of hydrated oxide of iron and sulphuric acid.

How far hydrogen sulphide and sulphides are capable of being directly oxidised by solutions of nitrates does not appear to have been sufficiently studied. There is no doubt that nitrates are rapidly reduced in presence of sulphide mud; how far this is a purely chemical change and, if so, what is the exact cause of the change, has not been fully determined.

Beyerinck claims to have isolated an organism, *B. thioparus*, which brings about the following decomposition :—

$$5S + 6KNO_3 + 2H_2O = K_2SO_4 + 4KHSO_4 + 3N_2$$

The most frequently occurring and obvious case of oxida-

tion of hydrogen sulphide by bacterial agency is that brought about by the higher bacteria, classified under the general term of *Beggiatoa* (Fig. 3 (I^A)). These are the organisms which form the subject of Wienogradski's researches above referred to. They are found very often in sulphur springs and wherever putrefying sewage or suchlike organic matter comes in contact with air, as, e.g., on the stones of a stream in the neighbourhood of a badly polluting discharge. The organism, as a matter of fact, grows between wind and water, but makes use of the sulphur either by decomposition of the H_2S present, or by actual absorption of the free sulphur formed by its spontaneous oxidation. If a strand of *Beggiatoa* is examined under a high-power microscope, very characteristic granules of sulphur are seen to be present throughout the organism, as is shown in Fig. 3 (I^A) (Chapter II). This sulphur is the amorphous form soluble in carbon bisulphide. *Beggiatoa* is capable of absorbing large quantities of sulphur which it oxidises to sulphates; for this purpose it is necessary that carbonates should be present in the surrounding liquid. Under its natural conditions of growth this will inevitably be the case, ammonium carbonate, e.g., being always present in decomposing sewage. *Beggiatoa* appears to use the sulphur as a source of energy rather than to increase its cell substance. Wienogradski found that it could use up from two to four times its weight of sulphur without increasing in growth. Under these circumstances, comparatively small amounts of organic matter will suffice to sustain it, and thus it can flourish in sulphur springs, whose chief constituents, apart from hydrogen sulphide, are mineral salts.

To summarise the contents of the foregoing chapter, we may conclude that sulphur enters the cycle of living nature as mineral sulphates in the food of plants. By the decomposition of vegetable albumin, or at a further stage from the excretory products of animals, it may reappear as hydrogen

sulphide (sulphuretted hydrogen). This may be re-oxidised to sulphates, either directly by chemical means, e.g., oxides of iron, etc., or by the intervention of bacteria. Certain of these oxidise it directly to sulphate, while others make use of the presence of nitrates.

Sulphates are capable of being directly reduced to hydrogen sulphide by certain bacteria, in presence of small quantities of organic matter, but such changes only take place in absence of air. These various changes clearly indicate the importance of abundant supplies of oxygen, if the evolution of hydrogen sulphide, and the other less well-defined objectionable gases which accompany it, are to be avoided. The bearing of this principle on the purification and disposal of sewage and other waste organic matter, will be further referred to in Chapter XVII.

CHAPTER XV

FERMENTATION OF INDIGO, TEA, COCOA, COFFEE, AND TOBACCO

Indigo.—The important series of researches carried on during recent years on behalf of the Government of India on the chemistry of natural indigo, and of the native processes of manufacture, is of especial interest to the student of enzyme chemistry.

As with most native industries, a considerable amount of empirical knowledge and skill has been attained in the manufacture of indigo, through centuries of experience, and the improvements to be effected do not usually lie on the surface, although at first sight they may appear to do so. Scientific research of a high order is requisite, together with special knowledge of local conditions, if a real gain in efficiency is to be achieved.

It is partly for this reason that native methods of manufacturing indigo have been practically stationary for many years. The author has recently seen indigo vats (Plate III (i)) near Mirzapur, U.P., whose construction and methods of use do not greatly differ from the graphic description to be found in a volume, ' Rural Life in Bengal,' published in 1860.

The native method for extracting indigo from the indigo plant is briefly as follows :—

The plant is brought to the factory immediately after cutting and placed in bundles in an upper series of stone or concrete vats. The bundles are tightly pressed down

by means of bamboos, and heavy baulks of timber levered down and fixed in position by horizontal pins, passing through two uprights at each end of the vat. The vats containing the pressed bundles are filled up with water and steeping is continued over night, the liquor being allowed to run off into lower vats in the morning. The liquor in the lower vats is then thoroughly beaten up by men who stand immersed to the hips in the liquor and beat it with bamboos shaped like oars, or artificial beaters of various kinds are used. The object of this process is to bring the liquor thoroughly in contact with air when the indigo is precipitated. The progress of the operation is tested by the manager by inspection of small portions of the liquor from time to time.

On completion of the beating process, the indigo is allowed to deposit, the liquid run off to waste, and the wet indigo mud run on to draining cloths. When it has attained a suitable consistency, portions are wrapped in cloth and pressed like cheeses in a press; the pressed mass is then cut into cakes and finally dried.

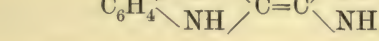
The impetus towards improvement of this process has been due to the acute competition during recent years of artificial indigo, the extent of which may be gathered from the fact that in 1896, out of a total weight of 46,683 cwts. of indigo imported into Great Britain, only 7,641 cwts. consisted of the natural product.

In 1902 Mr. W. Popplewell Bloxam and his colleagues began their researches for the Government of Bengal. The work was carried on in India for two years and was afterwards continued from 1905 to 1907 in the University of Leeds, under the general supervision of Mr. A. G. Perkin, F.R.S.

A report of this work was published in 1908 by the Government of India, and the following information is mainly taken from its pages.

In the first place it should be explained that the pure colouring matter of indigo is *indigotin*, which has the molecular

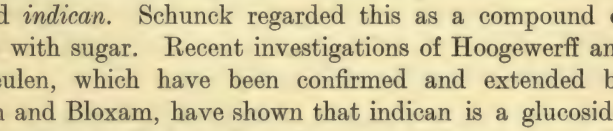
formula $C_{16}H_{10}N_2O_2$. Careful study of its related products leads to the following structural formula for indigotin which was first prepared artificially by Von Baeyer in 1878 :—

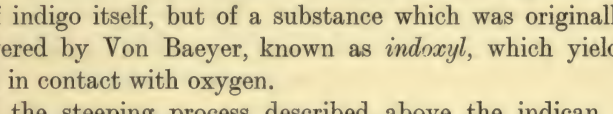


It is to Von Baeyer and his pupils that we owe the knowledge of the structure of indigo, which has rendered possible its commercial production on the large scale from raw material, such as naphthalene, found in coal-tar.

Indigo does not exist as such in the indigo plant. Schunck in 1855 showed that the plant contained a *glucoside* which he termed *indican*. Schunck regarded this as a compound of indigo with sugar. Recent investigations of Hoogewerff and Termeulen, which have been confirmed and extended by Perkin and Bloxam, have shown that indican is a glucoside, not of indigo itself, but of a substance which was originally discovered by Von Baeyer, known as *indoxyl*, which yields indigo in contact with oxygen.

In the steeping process described above the indican is fermented, yielding indoxyl and glucose ; in the subsequent beating operation the indoxyl is oxidised. The equations representing these changes, supposing them to be complete, are as follows :—





Perkin and Bloxam's researches were concerned with the exact study of these chemical changes. Before this was possible, accurate methods of analysis had to be devised in

order to determine the amount of indigotin in the cake indigo, and of indican in the original plant, older processes all giving conflicting results. The methods finally devised were briefly as follows :—

For the determination of indigo, one gram of indigo was converted into a tetrasulphonate by means of fuming sulphuric acid, and the tetrasulphonate precipitated as potassium salt by addition of potassium acetate. The precipitated salt can be filtered off, dissolved in water and oxidised by potassium permanganate of known strength.

The determination of the *indican* in the leaf depends on the fact that, when brought into contact with a substance known as *isatin*, a pure crystalline compound known as *indirubin* is formed, by the combination of indoxyl and isatin, according to the following equation :—

$$\underset{\text{Indoxyl}}{C_8H_7ON} + \underset{\text{Isatin}}{C_8H_5O_2N} = \underset{\text{Indirubin}}{C_{16}H_{10}O_2N_2} + H_2O$$

It was found that the best method of extracting the indican from the leaf was by means of acetone.

Armed with these exact methods of analysis, Perkin and Bloxam have been able to show that the yield of indigo obtained in the native process by no means corresponds with the theoretical yield which should be obtained on the basis of the indican present in the leaf. Several by-products are present in natural indigo, particularly *indigo brown*, the investigation of which indicates that it is formed by a secondary reaction from indican. Their examination of a specimen of leaf from an indigo-yielding plant from Sumatra has shown that, under certain conditions, twice as much indigo may be present, as in the best leaf from Java.

The decomposition of indican they agreed to be due to the action of an enzyme present in the leaves, rather than to the activity of bacteria. In this they confirm the opinion of other investigators, notably Beyerinck, Bergtheil, and Rawson. They agree with Beyerinck that the enzyme is

insoluble in water. The exact character of this enzyme, and especially its conditions of formation in the plant, afford material for further study. According to Beyerinck it is not an oxidase, nor has he been able to find this class of enzyme in the indigo plant.

As a result of all these researches improvement in the present method of native indigo production is to be sought along the following lines of investigation :—

1. New plants such as the *Indigofera sumatrana*, giving a greater yield of indigo, may be introduced.

2. The study of seasonal variation in the percentage of indican in the plant may result in an increased yield.

3. The effects of manuring may be further studied, with special attention, it may be, to the organisms in the soil. The indigo plant is leguminous, and possesses root nodules, which also call for investigation.

4. The accurate control of the beating or blowing operation.

In view of the researches of Brown and Morris, it might even be suggested that the time of day at which the plant was gathered would condition, to some extent, the proportion of indican present in the leaves ; and the suggestion made by several workers that the leaves rather than the whole plant should be plucked would seem to be worth attention.

It must always be remembered, when comparing what are generally called natural processes with artificial methods, that *prima facie* the advantage lies with the natural method, which depends on the inexhaustible energy of the sun's rays ; when this advantage is coupled with cheap labour and scientific control, such a native process should be able to stand considerable competition. It is the scientific control which up to recent years has been lacking, and it may be hoped that, for social and economic reasons, these researches will be successful in maintaining an industry which gives healthy and satisfactory employment to a large number of people.

Tea.—Tea is produced in two forms for the market, viz., green tea and black tea. In the manufacture of green tea the object is to maintain the colour and to prevent fermentation; the leaf is therefore roasted immediately after picking and the whole process of manufacture conducted as quickly as possible. In the case of black tea the leaves are dried slowly, and in the course of the process fermentation takes place. This fermentation is a special feature in the formation of Indian black tea, and has been the subject of very interesting researches by Dr. H. H. Mann, to whom the author is indebted for the special information of this section.

The following processes are involved in the manufacture of Indian black tea :—

1. *Withering.*—Withering of the leaf, which consists in exposure to the sun on fine basket-work trays.

2. *Rolling.*—Rolling by machine, which has the effect of pressing out a certain amount of the juice of the leaves. The soft leaves are often made into balls which are used to absorb the juice.

3. *Fermentation.*—These balls are broken up and allowed to *ferment* and then spread out to dry in the sun.

4. *Firing.*—This takes place in a chest of shallow firing drawers, the bottoms of which are made of fine wire gauze.

5. *Sorting.*—In this process various qualities of leaf are sorted by sieving, etc.

Dr. Mann's researches have been concerned primarily with the changes going on during the withering and fermentation processes, and the relation of these to the quality of the tea.

The quality of tea appears to depend on the following factors :—

(*a*) *The flavour*, caused principally by an essential oil.

(*b*) *Pungency*, caused in greatest measure by the unfermented tannin.

(*c*) *Colour* of liquor, caused chiefly by the fermented tannin,

(*d*) *Body of liquor*, measured principally by the total soluble matter, of which a large part is tannin both fermented and unfermented.

It was found that the fermentation is the result of enzyme action ; the presence of bacteria during the fermentation process is distinctly injurious, rendering the tea sour and unfit for consumption. In order to prevent deleterious changes of this sort, it is necessary that the fermentation should be carried on under aseptic conditions, that is, scrupulous cleanliness must be maintained throughout the process. The use of antiseptics is injurious to the enzyme as well as to the micro-organisms. If the temperature also is kept at about 80° F. the change is found to be mainly enzymic. The chemical change which takes place during fermentation consists essentially in an oxidation of the tannin. It has been found indeed that there are two enzymes present; one of these colours guiachum resin blue at once, the other does so only in presence of hydrogen peroxide. The main ferment is an oxidase, causing the darkening of tea juice and also of pyrogallol and hydroquinone. It has been found that the flavour improves in proportion to the amount of enzyme in the leaf. It would appear that in the tea leaf the tannin is combined with sugar ; during fermentation this compound is split up and the tannin is oxidised to brown products. This oxidised tannin combines with other substances in the leaf-forming compounds, some of which are insoluble in water ; there is, therefore, a decrease in soluble tannin. It is possible for this to go too far and the pungency of the tea to be injuriously affected.

The enzyme increases during the withering of the leaf, and one of the most important results of Dr. Mann's investigations is the possibility of the exact control of the withering process. The object of withering is twofold—to soften the leaf in preparation for rolling, and to produce the greatest amount of enzyme. Under normal conditions these two changes are practically simultaneous, but in very dry weather the leaf

may be physically ready to roll before sufficient enzyme is developed; and on the other hand, in very wet weather, the leaf may be chemically ready for rolling before it is properly withered. It may be possible, therefore, to control the time of withering, either retarding it by heaping up the leaves or quickening it, e.g., by means of fans, and so obtaining the necessary conditions for the production of the best tea.

It is of further interest that the amount of enzyme in the leaf has been shown to depend on the percentage of phosphoric acid used in manuring the plants; further, much more enzyme is present in leaves plucked at 6.30 A.M. than at 6 P.M., which supports the suggestion made with regard to the indigo plant in the preceding section.

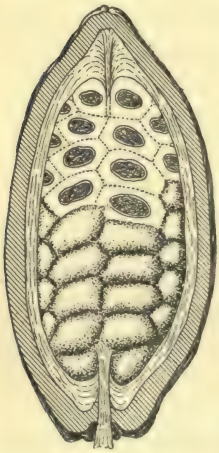


FIG. 25.—COCOA FRUIT IN PART SECTION.

The Fermentation of Cocoa.—Cocoa, as known to the consumer, is obtained by grinding and roasting the seeds or beans of the cocoa fruit; the appearance and structure of the latter can be understood by reference to Fig. 25. In order to obtain the beans free from surrounding pulp, a process of fermentation is resorted to. The fresh beans, after separating them from the shell, are piled on a floor or filled into boxes, and allowed spontaneously to ferment. Plate III (ii) gives an idea of the appearance of these fermenting boxes. A period of two to six days, according to circumstances, is usually allowed for fermentation. A rise of temperature, amounting to about 5° C., takes place in twenty-four hours, and in the course of four days the fermenting beans may have a temperature as much as 18° to 20° C. above the surrounding atmosphere.

PLATE III.



[*Photo by Author.*

(i) Indigo Vats near Mirzapur, India.



[*Photo lent by S. H. Davies, M.Sc.*

(ii) Fermenting Boxes for Cocoa.

The chief purposes of the fermentation process are [1] :—

1. To arrest the germinating power of the seed;
2. To remove or contract the pulp surrounding the seed;
3. To loosen the connection between the seed and its testa;
4. To develop the colour of the bean and to improve the taste of the cocoa.

The separation of the pulp is originally due to the activity of yeasts, which develop in the sweet juice oozing from the pulp; an alcoholic fermentation takes place in the inner portions of the mass, which gives place to an acetic fermentation in those portions in contact with air. These changes result in an elevation of temperature and a considerable discharge of acid juice, which is sometimes used as vinegar. At the same time the beans become loosened from their surrounding integument, from which they can afterwards be easily separated by washing.

The bean in its fresh state has a violet colour; on exposure to air the violet colour changes to a deep brown. The change of colour from purple to brown takes place to some extent during the fermentation process, and is completed in the subsequent drying. It has been shown that this change of colour is due to the action of an oxidase in the cocoa bean. If the bean is boiled or treated with acid, no change of colour can afterwards be produced, showing, therefore, that it is due to the action of an enzyme. It appears that both an oxidase and a peroxidase are present. Thus, if a freshly cut bean is moistened with tincture of guiachum, a blue colour is rapidly produced, indicating the presence of an *oxidase*. If the bean is crushed with a little water and heated for five minutes to 75° C., no coloration is given with guiachum, showing that the oxidase is destroyed at this temperature. On addition of

[1] See *The Fermentation of Cacao and of Coffee*, by Dr. Oscar Loew, published in the Annual Report of the Porto Rico Agricultural Experiment Station for 1907, to which the author acknowledges his indebtedness in the present and the succeeding sections.

hydrogen peroxide to the unfiltered juice, a blue colour is obtained, but is not developed in the juice after filtering. This indicates the presence of an insoluble *peroxidase*.

The flavour of the cocoa appears to be improved by the fermentation process, probably in consequence of the partial oxidation of a tannin present in the bean, but some difference of opinion exists on this point. The flavour is chiefly developed in the subsequent roasting, but the action of the oxidases would seem, from the colour produced, to be a necessary preliminary to this process.

The Fermentation of Coffee.—The coffee fruit, whose structure is illustrated in Fig. 26, is subjected to a fermentation similar to the one above described in connection with cocoa; chiefly in order to loosen the seeds from their surrounding integument. The essential part of this process is a solution, apparently by enzyme action, of the adhesive substance between the parchment envelope and the slimy layer, so that after the fermented coffee is washed and dried, the parchment becomes brittle and is removed, together with the silver skin, in the process of coffee milling. This last process is frequently done in London, and not in the country where the coffee is produced. The effect, if any, of the fermentation process upon the flavour of the coffee has not hitherto been fully investigated.



FIG. 26. COFFEE BEAN.

Tobacco.—The curing of tobacco is again a fermentation process. The leaves after gathering are first slightly withered, then 'sweated' in moderate-sized heaps, and finally fermented in large heaps containing as much as fifty tons of tobacco.

It has been considered that this fermentation is a bacterial process, and pure cultures have even been introduced in order to impart specific aromas to the tobacco. More recent

researches by Loew and other chemists of the United States Board of Agriculture, lead to the conclusion that the changes are essentially due to enzyme action; oxidases and peroxidases have been detected, and especially a soluble and insoluble catalase, an enzyme capable of decomposing hydrogen peroxide. The changes taking place in the curing of tobacco consist, in the first place, in the elimination of starch and sugar, by the continued respiration of the plant cells during drying. The ethereal extract and the percentage of tannin also decrease. During the fermentation the nicotine also decreases, and the colour and aroma improve. The effect of the character of the soil on the quality of tobacco is, of course, well known, and greater control of the quality, in parts of the world which hitherto have not yielded the finest brands of tobacco, must be sought in investigations similar to those which have been described in connection with indigo and with tea.

CHAPTER XVI

BACTERIOLOGICAL AND ENZYME CHEMISTRY IN RELATION TO AGRICULTURE

It is becoming increasingly necessary for the scientific agriculturist to be well acquainted with the chemical changes induced by bacteria and by enzymes. The economical use of farmyard manure is better understood by a knowledge of the character of fermentation which it undergoes, both spontaneously and in contact with the soil, before it is fitted for the food of plants. The conditions of fertility of soils, including the maintenance of a sufficient proportion of nitrogen, are intimately related to the bacterial life of the soil.

For a right understanding of the conditions of growth of plants, careful study is required of the changes brought about by enzyme action in the various organs of the plant, particularly in the seed and leaves.

Important enzyme changes also occur in the preparation of special fodder or silage for stock.

Finally, for successful dairy work, especially the ability to maintain a constant quality in butter and cheese, a knowledge of bacteriological chemistry is now almost essential. In the following pages these aspects of the subject will be briefly dealt with in order.

Farmyard Manure.—Stable manure is of course a complex mixture of substances, and the possible fermentations which it may undergo are very various. When the animals

are kept in the fields, manure is returned directly to the ground, and gradually becomes broken down therein. It is when manure is collected from stables and stalls that considerable loss may occur, if care is not taken. Farmyard manure consists of dung and urine, mixed with straw or other material used for bedding, such as peat-moss litter, etc. The dung will contain the undigested portions of the animals' food, together with a certain amount of waste material from the digestive organs. The more valuable portion of the nitrogenous output of the animal is in the urine. The main fermentations, therefore, that will take place in stable manure are :—

1. Ammoniacal fermentation of urine and of hippuric acid ;
2. The breaking down of albumin derivatives ;
3. The decomposition of carbohydrates and especially of cellulose.

All of these have been referred to in previous chapters ; it is only necessary here to indicate their practical bearing.

It is clear, in the first place, that every care must be taken, if the full value of the manure is to be obtained, that the urine is not allowed to run to waste ; for this reason stables and yards should be well paved and the manure should be kept on an impervious floor. Another less obvious cause of the loss of nitrogen from manure, apart from the actual running to waste of the liquid portions, arises from the volatilisation of ammonia, owing to the dissociation of the ammonium carbonate, formed by ammoniacal fermentation. This loss is greatest when the manure is fresh, as ammoniacal fermentation is almost the first to set in ; later on acids are formed by the decomposition of carbohydrates and cellulose, which tend to fix the ammonia. One advantage of the use of peat-moss litter is that it has the power of retaining ammonia. It has been found, however, by the experiments of Dehérain and others, that if care is taken to pile the manure heap in such a way as to exclude air, the CO_2 evolved by various fermentations prevents the dissociation of ammonium carbonate and consequent

loss of ammonia. Following the ammoniacal fermentation will be the decomposition of albuminoids, yielding ultimately, as has been shown, various amino acids. The decomposition of carbohydrates, other than cellulose, which occur in dung, e.g., starch, gums and possibly certain sugars, will also occur with some rapidity, yielding acids capable of uniting with ammonia and any other bases present; these various decompositions take place with considerable evolution of carbon dioxide.

The fermentation of the *cellulose* is the longest delayed, and probably takes place both anaerobically and aerobically, according to the conditions obtaining in different parts of the manure heap. Acids are also produced here as by-products; probably also the valuable residual *humus* is a product of the fermentation of cellulose.

The quantity of nitrogen in the manure will depend, as already indicated, on the care taken to exclude air in the manner of forming the manure heap. If the heap is well pressed down, the conditions are mainly anaerobic, and the heap can be kept for considerable periods without serious loss of valuable constituents.

It is sometimes necessary, e.g., for market gardening, to prepare manure quickly, and large piles may not then be conveniently made. In such a case a considerable quantity of nitrogen passes off in the free state, apparently by direct oxidation of nitrogenous matter.

Well-rotted manure will contain all the materials for plant food, and the time which has elapsed in its preparation will be saved by the greater availability of its constituents when it is placed on the ground. According to Warington one ton of farmyard manure supplies 9 to 15 lbs. of nitrogen, a similar amount of potash, and 4 to 9 parts of phosphoric acid. It is thus, of course, an attenuated manure, and further changes have to take place after it is incorporated with the soil, before the plant can make full use of it; the

physical character of the soil is, however, improved by its presence. The resistant portions of fibre and straw tend to make the soil more porous, and the humus which it contains increases the power of the soil to retain water and ammonia salts, and also improves the texture of the soil.

After the manure is placed on the field the various amino compounds will suffer further decomposition, yielding eventually ammonia. It is a matter of some uncertainty whether ammonia is immediately available for plant food; at any rate, there is no doubt that nitrates are more readily taken up by a plant, and, therefore, a prolonged retention of nitrogen compounds in the soil, and their slow conversion into ammonia, and finally into nitrate, is an advantage. As a matter of fact, nitrification of the ammonia generally takes place before it has been removed from the soil by the plant. Moreover, the weight of the dry matter of the plant increases per unit of nitrogen, supplied as nitrate.

The conditions of nitrification of ammonia, whether supplied as stable manure, or in the various forms of artificial manure, especially sulphate of ammonia, have been considered from the laboratory point of view in Chapter XIII; the importance of the presence of humus has just been noted. It will be further clear, upon consideration, that nitrification will not take place satisfactorily in soils which have become sour or acid; an addition of *lime* is necessary in such cases. The beneficial effect which unquestionably attends, in many cases, the use of pressed sludge, which contains only small quantities of nitrogen relatively speaking, is due to the presence of lime, which has been added to facilitate the operation of pressing. The physical effect of the admixture of such material with the soil is of importance, and the presence of a certain proportion of matter of the nature of humus is also beneficial.

The Loss and Recovery of Nitrogen in the Soil.—It is

evident that a crop, such as wheat, of a high nitrogen content, must diminish the supply of this element in the soil, and if such a crop is continually grown on one plot, the nitrogen must become exhausted unless replaced in various ways. Besides the loss of nitrogen from cropping, other sources of loss occur by drainage ; the nitrates pass away in the subsoil water, and heavy rainfall on a porous soil accelerates this loss. A further source of loss has been referred to in Chapter XIII, viz., the elimination of nitrogen from nitrates by the de-nitrifying organisms. Against these sources of loss of nitrogen have to be set the following sources of gain, apart from the application of nitrogenous manure. A certain amount of nitrogen is added to the soil in rain, though, as already explained, this may wash out more than it brings. It is to the nitrogen-fixing bacteria that we have largely to look for the economic maintenance of the balance of nitrogen ; and we have here an explanation of the advantage of growing leguminous crops at intervals. If, after a succession of nitrogen-exhausting crops, such as wheat, a crop of clover be grown, and the stubble afterwards ploughed in, the nitrogen content of the soil is greatly increased. This is due, as has been explained, to the action of organisms, which find their habitat in the root nodules of leguminous plants, such as the clover, which in some way enable the plant to obtain a store of nitrogen from the air.

Experiments on a small scale have shown that it is possible greatly to increase the growth of such plants, when grown in sand, by inoculating the sand, or the seeds of the plants, with suitable cultures of nitrogen-fixing bacteria. Attempts have been made to carry out this process on the large scale. The best results have been obtained with species of leguminosæ introduced into a country for the first time, e.g., the soy bean in the United States and Germany, lucerne in Scotland, and certain non-indigenous plants in Canada. For crops which have already long been cultivated, e.g., clover in England, the conditions of success do not so far seem to be fully understood.

Fertility of Soils.—Enough has been said to show the great importance to the farmer of the bacterial life in the soil. Dr. E. J. Russell has carried out important investigations showing that the fertility of the soil is, under normal circumstances, actually proportional to the bacterial activity of the soil. It is, of course, obvious that bacterial activity is a very wide term, and covers the many classes of action which have been indicated in the foregoing paragraphs, but Dr. Russell has found that the sum of these activities can be measured, by determining the rate at which oxygen is taken up by a given weight of soil, and also the total amount of oxygen so taken up. This he determined by enclosing the soil in a flask, connected on one side with a tube dipping into mercury, and on the other with a small receptacle containing strong potash solution, which served to absorb the carbon dioxide produced by the oxidation of the organic matter. The rise of the mercury in the side tube enabled the rate and amount of oxygen absorption to be measured. A number of these flasks, each containing soil, whose character as regards fertility was known, was placed in a common water-bath, and maintained at a constant temperature, one flask being left empty to serve as control. It was found, as already stated, that the absorption of oxygen, and consequently the bacterial activity, increased with the fertility.

In the face of these results, it appears surprising that experiments, by Russell and others, should have shown that partial sterilisation of the soil, either by antiseptics, such as toluene, or by heat, should increase the fertility.

An explanation of this apparent contradiction is afforded by a recent research by Russell and Hutchinson. They effected partial sterilisation either by heating to 98° C., or by addition of 4 per cent. of toluene, which, at the end of three days, was allowed to evaporate by spreading out the soil in a thin layer. In a third series, the toluene was left in; in a fourth series, the soil was left untreated. The soils were

moistened, and kept for definite periods in bottles, stoppered with cotton wool, at the ordinary laboratory temperature. Determinations were then made :—

(*a*) Of the production of ammonia ;
(*b*) Of the production of unstable nitrogen compounds ;
(*c*) Of the proportion of humus ;
(*d*) Of the nitrification ; and
(*e*) Of the total amount of nitrogen.

The effect of partial sterilisation was found to be :—

(1) An increase in the amount of ammonia ;
(2) Cessation of nitrification.

Besides the chemical observations, they determined the total number of bacteria ; and they found that the increased ammonia production, due to partial sterilisation, was accompanied by an increased number of bacteria. The problem resolves itself into finding out why the bacteria increase so much more rapidly in the partially sterilised than in the untreated soil. They found that if untreated soil were added to partially sterilised soil, the rate of ammonia production was reduced, but this was not the case if an extract of the untreated soil, filtered, but still containing bacteria, was added to the partially sterilised soil. This would indicate that the inhibiting agent was something which affected bacterial growth, but which could be removed by a coarse filter. Such an agent would be found in large organisms capable of feeding upon bacteria. As a matter of fact, upon examination, many of these were found in the untreated soil.

Russell and Hutchinson therefore conclude that the large organisms, that is, protozoa of various kinds, are an important factor in limiting the bacterial activity, and therefore the fertility of untreated soil. When toluene is added to the soil, or when the soil is heated to 98° C., these phagocytic, or bacteria-consuming, organisms are destroyed, but the bacterial spores are not. On removing the toluene, and adding moisture, the spores germinate, and the other bacteria

multiply with great rapidity, since they are now free from the attacks and the competition of their enemies, the other large organisms. The dead organisms, in fact, were shown to afford food for the bacteria.

It was further found that plant growth increased in partially sterilised soil, although nitrification was inhibited; under these conditions it appears that the plants can obtain their nitrogen from a source other than nitrates.

These experiments are of the highest interest, and show that much remains yet to be discovered with regard to the conditions of bacterial life in soil, and its relation to the growth of plants.

Chemical Changes in Plant Cells.—When a plant is burnt, its organic constituents disappear, mainly as carbon dioxide, CO_2, nitrogen and water, H_2O; its mineral constituents remain behind in the ash. The growing plant builds itself up again out of these products of its combustion; the mineral constituents and water it takes in through the roots, the carbon and oxygen through the leaves, the nitrogen ultimately being supplied from the sources already discussed. All the complex physical and chemical processes involved in building up a plant are controlled ultimately by the vital energy of the plant cells, together with the energy of sunlight. The correlation of all these processes is the task of physiological botany, and a knowledge of this is obviously indispensable, if the plant is to be grown under the best conditions and supplied with its right food. Enzyme chemistry forms the foundation knowledge of physiological botany. It is clearly necessary to have some understanding, in the first place, of the primary chemical changes taking place in specific cells, before establishing their general relations.

The initial impulse to plant growth is to be found in the potential biotic energy of the seed, or, more properly speaking, of the embryo. This, of course, like all forms of life that we

are acquainted with, has its origin in pre-existent life, but the distinction between the changes taking place in the seed, and those which occur in the leaf, is that the former are not directly dependent upon sunlight, unless indirectly, it may be, for warmth.

The chemical changes taking place have been fully illustrated in the study of the barley grain, to which Chapters V and VI have been devoted. The seeds of all plants contain in the endosperm a store of reserve material, which has been elaborated by the growing plant. The embryo, as we have seen, has the power of secreting various enzymes, viz., *cytase*, which breaks down the cell walls of the endosperm, and *amylase*, which converts the starch into sugar. There are also present *proteolytic* enzymes, which break down the stored albumin of the seeds, and, in the case of fat-containing seeds, such, e.g., as those of the castor oil plant, *lypolytic* enzymes are present, which break down the oils or fats. All these changes, it may be seen, are essentially concerned with the breaking down of material already elaborated, i.e., they are what is known as *catabolic*; unless fresh nutriment is supplied, on the one hand, and fresh energy on the other hand, growth will cease. Nutriment is supplied to the plant, as already stated, by the roots, and by the leaves; energy is supplied by the leaves only, and it is in the leaf cell that we have to look, to find what we may describe as the power house of the plant.

The chemistry of the leaf cell is one of the most fascinating problems which has occupied the attention of chemists, but in spite of numerous researches by highly qualified workers, it is still very imperfectly understood.

The simple beginnings and ends of the process have been known for a long time; they may be studied without difficulty, and indeed form one of the subjects of most 'nature study' classes. The following experiments are easily carried out.

A portion of American pond weed, *Elodea canadensis*, is

placed in water, in a cylindrical vessel, with a little earth at the bottom for root attachments, and the whole set in the sun; bubbles of gas soon arise from the leaves and may be readily collected. If a glowing splinter of wood be held in the gas, it will burst into flame, showing that the gas consists, for the most part at any rate, of oxygen.

On the other hand, if the plant is placed in darkness, and air, freed from CO_2 by passing through potash solution, is led over the plant, and then passed into baryta water, the latter will become turbid from formation of barium carbonate.

From these experiments, it is clear that two main processes go on in the leaves, the evolution of oxygen in sunlight, and of carbon dioxide in darkness. These two changes, as a matter of fact, take place at all times, but the preponderance of one over the other depends on the presence or absence of sunlight. The evolution of oxygen is a building up, or *anabolic* process, arising from the decomposition of the carbon dioxide in the air, the plant utilising the carbon and giving off the oxygen; on the other hand, the evolution of carbon dioxide is essentially a process of respiration, or a *catabolic* process, where the carbonaceous constituents of the plant are broken down, with production of carbon dioxide and water. The volume of the oxygen given out in the assimilation process is practically equal to the volume of carbon dioxide taken in, sufficiently indicating that the changes involved are of a fairly simple order. The problem to the chemist is to discover how the carbon, taken in by the plant as CO_2, is built up into starch and cellulose, and by what stages these latter are reconverted into carbon dioxide, and thus the life cycle maintained. Microscopic observation indicates that starch is the first visible product appearing in the leaf cell; but, of course, between a simple substance such as carbon dioxide and starch, the chemical steps must be very numerous.

In 1870 Von Baeyer put forward a very suggestive hypothesis in regard to the first of these steps; the simplest carbohydrate,

as was explained in Chapter IV, is formaldehyde, CH_2O. Von Baeyer suggested that, in the simultaneous presence of light and of chlorophyll, carbon dioxide and water may react according to the following simple equation :—

$$CO_2 + H_2O = CH_2O + O_2 :$$

An explanation is here indicated of the equivalence alluded to above between the CO_2 decomposed and the oxygen evolved. It is easy to conceive further, that the formaldehyde, by a series of polymerisations, can build up more complex carbohydrates, such as starch. This hypothesis derives confirmation from the fact that, on standing in contact with a dilute solution of lime water, formaldehyde does, as a matter of fact, become gradually converted into a mixture of hexoses. Until recently, however, all attempts to realise the formation of formaldehyde from carbon dioxide and water in the laboratory were without success, formic acid being always the product of the reaction. Nor was it possible to detect formaldehyde in the living cell.

Recently, however, the subject has been advanced considerably by the investigations of Usher and Priestly ; they have been able to show that if leaves of Canadian pond weed, *Elodea canadensis*, and certain green seaweeds, viz., *Ulva* and *Enteromorpha*, are first placed in hot water, so as to kill the protoplasm, and are then exposed to moist carbon dioxide in presence of light, formaldehyde and hydrogen peroxide are produced, and can be detected. If a suitable *catalase*, or hydrogen peroxide decomposing enzyme, were introduced into the mixture, oxygen was evolved.

Under the conditions of the experiment, when a certain amount of hydrogen peroxide and formaldehyde had been formed, a reverse change tended to be set up, the reaction being expressed as follows :—

$$H_2CO_3 + 2H_2O \rightleftarrows CH_2O + H_2O_2$$

Such a reverse change would not take place in plants, inasmuch as the products of the first reaction, formaldehyde and oxygen, are eliminated, the formaldehyde being utilised for building up carbohydrates, and the oxygen passing off.

We have still here to do with chlorophyll, a substance elaborated by life processes. Experiments by Fenton in 1907 have, however, shown that carbon dioxide can be reduced to formaldehyde in presence of metallic magnesium. This experiment is of interest in view of the fact that, according to Willstätter, magnesium is an essential constituent of chlorophyll, just as iron is an essential constituent of the hæmoglobin of the blood. It may be that we have here the first chemical step in the series leading up to starch.

The conversion of CO_2 into formaldehyde and oxygen would thus appear to be a purely chemical phenomenon, which under the conditions of the laboratory quickly reaches a limit, but which under the influence of biotic energy becomes continuous, owing to the products of the reaction being quickly removed.

This important first stage in plant assimilation may be expressed by the following equations :—

i. $HO-COOH$ (Carbonic acid) + $HO-H$ (Water) → $HO-HO$ (Hydrogen peroxide) + $COOH-H$ (Formic acid)

ii. $HO-CHO$ (Formic acid) + $HO-H$ (Water) → $HO-HO$ (Hydrogen peroxide) + $CHO-H$ (Formaldehyde)

The question still remains, presuming that the above equations bear a close relation to actual fact, what is the next stage between the simple carbohydrate, formaldehyde (CH_2O), and the more complex sugars and starches ?

It might naturally be assumed that the first detectable products would be simple sugars, such as bioses and trioses; such trioses have not been found. On the other hand there is evidence that acids, such as glyoxylic acid, $CHOCO_2H$, and glycollic acid, CH_2OHCO_2H, do occur in the leaves of plants, and the interesting suggestion has been made that in the process of reduction of carbonic acid, groups such as CHO, CH_2OH, CO_2H, CHOH, etc., are formed, from which various combinations, acids, aldehydes and carbohydrates may be built up. At the same time certain of these compounds might combine with ammonia, produced, it may be, by reduction of nitrates, to form amino acids, the first products of albumin synthesis. Still the fact remains that these intermediate products are not at all readily identified, and the evidence as to their presence is conflicting.

The careful experiments of Brown and Morris, in their research on the chemistry of foliage leaves, already referred to, reveal the somewhat surprising fact that, in the case, at any rate, of the nasturtium leaves, which constituted the chief material of their research, the first product of assimilation is cane sugar. Their method of experiment was to take leaves which were gathered early in the day, and dried at once after plucking, and compare their sugar content with leaves which were left exposed to the sun for some hours after gathering, with leaves which were gathered later on in the day and immediately dried, and with others placed in the dark for some hours after plucking. The dried leaves were extracted with ether, to remove fat and chlorophyll, and a weighed portion of the residue then extracted with alcohol to remove the sugars; the alcoholic extract was rendered slightly alkaline with ammonia, to prevent inversion of the sugars by means of the vegetable acids, small quantities of albuminous matter and of tannin removed by lead acetate, and the mixture of sugars, in the clear extract thus obtained, carefully analysed by polarimetric and copper reduction methods, in successive stages, viz. :—

(*a*) At once;

(*b*) After treatment with invertase to hydrolyse the cane sugar; and

(*c*) After complete inversion by means of hydrochloric acid.

In this way cane sugar, dextrose, lævulose and maltose were determined, and it was found in every case that the leaves which had been exposed to light, under conditions where assimilation processes were in the ascendant, always contained cane sugar in greater proportion than any of the other sugars present.

Thus, to take one example, leaves picked at 9 A.M. on a dull morning yielded the following analysis :—

Starch	3·24
Cane sugar	4·94
Dextrose	0·81
Lævulose	4·78
Maltose	1·21

Leaves picked at 4 P.M. on the same day after seven hours of sun gave :—

Starch	4·22
Cane sugar	8·02
Dextrose	0·00
Lævulose	1·57
Maltose	3·62

It has been possible to synthesise in the laboratory all the various sugars isolated by Brown and Morris; but one important difference exists between the products formed in the laboratory, and those produced by the activity of the plant cell. In all cases where a synthesis is effected in the laboratory by purely chemical means, optically inactive derivatives result, that is, mixtures of right-handed and left-handed forms in equal proportions. It is of course possible, by methods

already described, to separate these mixtures into their optically active constituents, as well as to produce optically active compounds in the first instance, if an optically active substance is used as a starting point. Thus, e.g., cane sugar can be synthesised by comparatively simple reactions from glucose and fructose, but in all cases where an optically active body is obtained, the agency of life steps in at some point.

This important fact was clearly realised by Pasteur, whose words on the subject are worth quoting: 'To transform an inactive compound into another inactive compound, which has the power of resolving itself simultaneously into a right-handed compound and its opposite, is in no way comparable with the possibility of transforming an inactive compound into a single active compound. This is what no one has ever done; it is, on the other hand, what living nature is continually doing before our eyes.'

It is to the action of enzymes present in the living protoplasm, especially in chlorophyll, that we must look for this selective synthetic power of the plant cell. We have learnt, through the work of Croft Hill and others, that the action of an enzyme may show itself in a building-up or anabolic process, as well as in a breaking-down or catabolic process. We may perhaps conceive of the enzyme as a kind of framework into which the molecules must fit themselves, in order that a certain substance may be produced, either on the up or down grade of a chemical change. Thus, in the case of maltose, we can conceive the various atomic groupings setting themselves to form maltose, or, on the other hand, passing back through the same framework to form glucose, the hexose which, it will be remembered, is produced when maltase acts upon maltose. Similarly invertase may act as the framework for the building up or breaking down of cane sugar.

It is evident that our knowledge of this subject is still of a speculative character, but enough has been said to indicate the

extraordinary complexity of the phenomenon of cell chemistry, even in a region so comparatively simple as the synthesis of carbohydrates. Apart from the purely scientific interest of researches in this direction, it is permissible to expect that an extension of knowledge of the chemistry of plant assimilation will render it possible more exactly to adapt the food supply of the plant to its special needs, and thus to conduce to economy in plant cultivation.

The Preparation of Ensilage.—In order to obtain a store of succulent food, for use when, through severity of weather, or for other reasons, it is naturally unavailable, it is a frequent custom, especially in America, to resort to the operation of ensilage.

In this process the fodder, e.g., hay, beet, cabbage leaves, or green maize stems, is packed into what is termed a *silo*, which may be either a closely pressed heap, protected from weather by thatch, or a large air-tight receptacle, usually cylindrical in form, into which the mass is pressed.

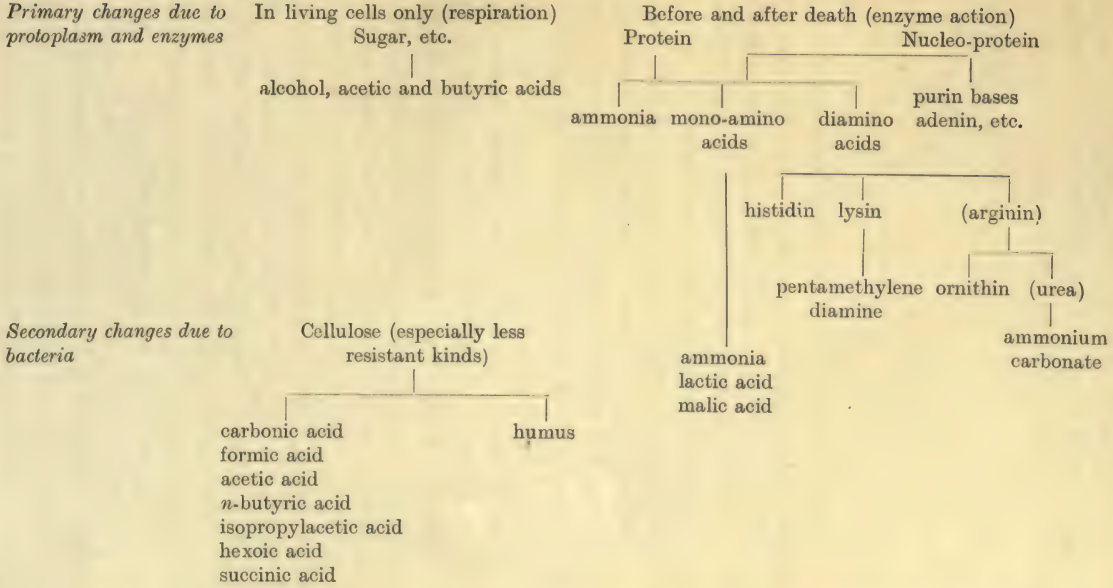
Under these circumstances fermentation sets in, accompanied by considerable heating. The heating is due to the continued respiration of the still living cells. In course of time the heat becomes such that most bacteria are killed, fermentation ceases, and the fermented fodder will keep for a considerable time. The character of the fermentation depends on the nature of the fodder used, and on the amount of oxygen and moisture present in the silo and in the original fodder employed.

Russell has carefully investigated the changes which take place during the ensilage of green maize. The vital processes of the cell protoplasm continue for some time after the maize is put in the silo, the starch continues to break down and the sugar formed is partially oxidised to various acids; in the limited supply of air, complete combustion to CO_2 does not take place. The proteolytic enzymes of the cell act on the

vegetable albumins, forming amino acids, etc.; these actions, being purely enzymic, will go on after the cell is dead. According to Russell, bacteria are also present, yet they are not the chief agents in the decomposition, though they probably attack the softer cellulose, producing humus and some fatty acids; they also carry to a further stage the decomposition of certain of the nitrogen compounds. These changes are summarised in the table on the opposite page, which may be taken as a typical statement of the changes occurring during the ensilage of fodder. In this particular case, maize was taken as the subject of experiment, and air was excluded as far as possible. With other materials, e.g. exhausted beet from sugar factories, etc., a greater proportion of acid may be obtained, in which case sour fodder is produced. It may even be possible to inoculate silos with selected ferments, in order to obtain the best results. Incidentally it may be mentioned that the 'sauerkraut' of the German restaurant is produced by an analogous species of fermentation.

The Bacteriological Chemistry of Dairy Products.—Starting from milk, as raw material, a great variety of products are obtained in modern dairy practice: cream of different flavours, butter both sweet and sour, cheeses in great variety, both soft and hard. These different products are not in general all produced in one dairy or even in one district; rather indeed are we accustomed to differentiate them according to the place of manufacture: thus Dutch cheese and Swiss cheese differ from those produced in England, and the different varieties of English cheese, as is well known, were at one time derived from different districts. The ultimate reason for this is to be found in the bacteriological conditions which long practice has established in various dairying centres. Modern advances in dairy practice seek to render it possible to produce any kind of dairy product at will, at any centre.

SCHEME SHOWING CHANGES DURING ENSILAGE



The substances in brackets are supposed to have been formed as intermediate products, though they could not be detected in the silage.

In order to understand these developments, the chemical constituents of milk must be first considered. According to Warington, cow's milk has the following composition:—

Water	87·0 per cent.
Albuminoids	3·7 „
Fat	3·9 „
Sugar	4·7 „
Ash	0·7 „

It is evident, therefore, that the possible chemical changes that may take place in milk are concerned with the decompositions of albumin which are brought about by proteolytic bacteria and enzymes, with the splitting of fats, and with the various fermentations which lactose or milk sugar is capable of undergoing. If these various changes are to be controlled, it will be seen that in dairy practice the utmost cleanliness is of the first importance, lest normal proteolysis should become putrefaction, or the breaking up of fats and of sugar should give rise to abnormal developments of butyric acid, and consequent rancidity, as distinguished from merely pleasant souring.

These considerations may first be applied to the *manufacture of butter*. Butter, as is well known, is obtained by the churning of cream, a process by which the fat globules, present as an emulsion in milk, are collected together to form the mass known as butter. The fat of milk consists largely of glycerides of palmitic and oleic acids, together with smaller quantities of the glycerides of other fatty acids, notably butyric.

The oldest method of separating the cream from milk is to allow the latter to stand in wide shallow dishes, when the fat particles, being specifically lighter than the rest of the milk, rise to the surface, and can be skimmed off. Such a process obviously offers conditions for contamination of the cream by air infection, especially if any carelessness is permitted. The danger of contamination is reduced if the cream is allowed to rise in deep closed vessels. But the modern process, in which

the cream is separated from the milk by centrifugal action in suitable machines, is the most rapid, and consequently the least liable to infection. If, at the same time, a low temperature is maintained, the danger of uncertainty in the subsequent souring of the cream is still further reduced.

If cream is churned in a perfectly fresh state, sweet butter is obtained, which is somewhat tasteless. By careful souring of the cream previous to churning, butter of a more defined flavour is produced ; it is in the control of this flavour that bacteria play their part.

In following the changes which they bring about, it must be understood that the cream, as used for churning, will contain not only the fat of the milk, but also a certain quantity of its other constituents. The composition of the butter is conditioned therefore by the method used for the collection of the cream.

The souring or ' ripening ' of the separated cream may be effected by the addition of a small quantity of sour milk, which will contain the necessary bacteria, notably the lactic acid bacteria. Satisfactory ripening of the cream can generally be judged by practice ; chemical examination of such cream should not discover any appreciable quantity of casein. Its presence would point to the souring process having gone too far, resulting in the production of a certain amount of clotting of the buttermilk present. Working in this manner, and with careful attention throughout to the avoidance of infection by deleterious bacteria, excellent results are obtainable so long as the conditions of milk supply and the general control of the dairy remain unchanged. An element of uncertainty, however, still exists, and the main reason for the greater sale of Danish butter, compared with that produced in England and Ireland, is its constancy of quality.

This constant quality has been attained by still further development in the control of the souring process.

Such absolute control is obtained when, in the first place,

all the bacteria present in the cream and its associated milk are destroyed by the process of Pasteurisation, and the subsequent inoculation of the Pasteurised cream with a pure culture of the necessary bacteria.

The process of Pasteurisation consists in a rapid heating to a temperature sufficient to destroy the majority, at any rate, of the bacteria present, followed by an equally rapid cooling process. By this method the composition and flavour of the cream is not appreciably altered. The pure culture to be added is generally known as the ' starter.' The following is the method described by the Danish bacteriologist Weigmann :—

A quantity of sweet milk, amounting to about two or three per cent. of the cream to be acidified, is heated to about 60° C. and quickly cooled ; to it is added a pure culture which is maintained by the addition of sweet Pasteurised milk from day to day. A portion of the inoculated milk is added to the cream, which is best prepared by cooling to a low temperature and then quickly warming up again to 16° or 20° C. The ripening process is generally started in the evening, and allowed to complete itself at a temperature of 15° to 20° C., the cream being ready for churning on the following morning.

The flavour of the butter produced depends on the particular starter used. It does not appear certain whether one variety of organism alone is concerned in producing specific flavours, although all the organisms concerned probably belong to the lactic acid producing species. At any rate one organism has been found by Professor Conn in America which produces a very excellent flavour. It was originally obtained from a specimen of milk from Uruguay, South America, exhibited at the World's Fair in Chicago. This bacillus is known as No. 41, and by its use constant results have been maintained on a very large scale.

Although, as has been stated, the ripening of cream is mainly produced by lactic acid bacteria, the precise chemical changes resulting in the production of a different taste or

aroma in different cases, is not very perfectly understood. Obviously the conditions admit of the production of esters, by combination of different organic acids with various alcoholic groups in a great variety of ways.

The chemistry of *cheese making* is more complex than the chemistry of butter making ; we have here, in addition to the activity of lactic organisms, to consider more especially proteolytic changes in the *curd*, which is the starting point of cheese. Reference has already been made to the chemical changes which take place when the clotting enzyme rennet is added to milk. The first process in the manufacture of cheese consists in throwing out the curd or casein by means of rennet. Milk can also be curdled by the activity of acid-forming organisms, and this method is actually employed in the preparation of certain home-made cheeses ; but in this case the curd is of a different composition, the whole of the albumin of the milk being thrown out. The curd produced by rennet, as we have seen, consists of casein together with calcium phosphate ; the curd will also carry down with it fat particles, and will retain, of course, a certain proportion of the *whey*, i.e., the liquid left after separation of the curd, and which contains the soluble constituents of the milk. Curd thus prepared is tasteless, and in order to be converted into cheese has to undergo a ripening process. In the process of cheese making the curd is granulated, placed in cloths, and the whey pressed out ; the pressed curd is then set aside to ripen.

The ripening process, in the case of cheese, is brought about, not only by bacteria, but also in certain cases by moulds. The former are chiefly concerned in the ripening of *hard* cheese, which may take place at a fairly high temperature, at which the activity of moulds will be inhibited. The activity of moulds is concerned more with *soft* cheeses, which are allowed to ripen at a lower temperature.

As in the case of butter, so in the case of cheese, if the

conditions of the dairy remain constant, a constant product may be obtained, without the necessary use of pure cultures. This constancy of conditions depends, however, on locality. Cheese making, e.g., is carried on in the high Alps under exceptionally favourable conditions in this respect; the air is pure, the fodder of the animals consists mainly of grass and hay, from fields to which they themselves contribute the only manure. In the lower Alps, on the other hand, the fodder, and consequently the manure, is subject to variation; the bacterial atmosphere, therefore, may change from time to time, and the variable conditions manifest themselves in the dairy.

The chemical changes taking place in ripening cheese consist:—

(*a*) In the breaking down of albumin;

(*b*) The splitting of fats;

(*c*) The fermentation of sugar.

That these changes are brought about by living organisms is clear from the fact that, if the curd is treated with an antiseptic, no ripening takes place. Numerous disintegration products of albumin have been detected in ripe cheese, such as leucin, tyrosin, and even ammonia. The splitting up of the glycerides is carried to a further extent than in the souring of cream; and the activity of the lactic organism is shown, in many cases, in the production of gas, which causes the pitting, so noticeable, e.g., in Gruyère cheese.

The control of cheese ripening by pure cultures is not so easily carried out as in the case of cream, since Pasteurised milk will not curdle with rennet. By initial care in the production of the milk and its maintenance at a low temperature, a reasonably pure curd can, however, be obtained.

The particular organism used as a starter will, of course, depend on the character of cheese to be produced; thus in the case of Roquefort cheese the mould *Penicillium glaucum* is used, whereas in the production of Cheddar cheese, lactic

acid bacteria have been shown to play the most prominent part.

In addition to being able to control the products of the dairy, the scientific dairyman must also understand the causes of the various abnormal and deleterious fermentations which may from time to time take place. These are mainly dependent on the invasion of so-called wild bacteria, whose nature and chemical activities have to be studied. It would lead too far to consider these difficulties here.

Although of recent years some attempts have been made in England to introduce scientific precision into dairy work, very much yet remains to be done. We are still very far from applying to the manufacture of dairy products the same standard of scientific thoroughness which has been so long worthily upheld in other fields of agricultural investigation, notably at the seventy-year-old experimental station at Rothamsted.

CHAPTER XVII

THE CHEMISTRY OF SEWAGE PURIFICATION

Owing to the general adoption of the water carriage system, together with the increasing scarcity of land in the vicinity of towns, great developments have taken place during recent years in so-called artificial methods for the purification of sewage. In order that works for this purpose shall be designed with due regard both for economy and efficiency, it is necessary that the changes, which it is intended to bring about by their means, should be thoroughly understood by those concerned in their construction. Although the actual construction of the modern sewage works is largely a matter of engineering, the design depends on careful adaptation of means to ends, and the bacteriological chemist and the engineer should here work in collaboration. The object of the works is to convert objectionable waste products, which if left to themselves would be a source of nuisance and danger, into other substances which are incapable of producing such objectionable developments. In the course of the necessary transformation, at one point or another, practically all the typical chemical changes, which have been considered in the theoretical chapters of this book, are met with, and the consideration of the processes carried on in a modern sewage works forms therefore an excellent illustration of the application of bacteriological and enzyme chemistry.

It will be necessary, in the first place, carefully to consider the composition of ordinary town sewage; for this purpose,

domestic sewage only will be referred to, the question of the treatment of trade effluents, or mixtures of trade effluents and sewage, constituting a special problem.

The main constituents of domestic sewage may be described as follows :—

(i.) MATTERS IN SOLUTION. (Mainly derived from urine.)
Nitrogenous substances, e.g., urea and kindred compounds.
Mineral salts, chiefly sodium chloride together with phosphates.

(ii.) MATTERS IN SUSPENSION, EMULSION, OR COLLOIDAL SOLUTION.—*Nitrogenous substances* of complex character containing sulphur (mainly derived from fæces).
Cellulose (disintegrated paper) and vegetable débris.
Soap and fat.

(iii.) SEDIMENTARY MATTERS.—Silt, clay, sand, etc.

There cannot, of course, be sharp lines drawn between these various classes of substances. It will depend, e.g., on the hardness of the water, how much, if any, of the soap is present in solution or suspension ; substances in Class ii. will also be partially carried down by the quickly sedimenting mineral matters of Class iii.

In general, about twenty gallons may be taken as the usual water supply per head per day, in which the above constituents are disseminated.

The complete purification of the sewage, which is effected by bacterial treatment, results finally in the production of some or all of the following substances :—

(i.) GASES :
Methane (Marsh gas).
Hydrogen.
Nitrogen.
Carbon dioxide.

(ii.) Soluble Salts :

Nitrates.
Phosphates.
Sulphates.
Chlorides.

(iii.) Insoluble residual matters conveniently termed '*Humus*.' [1]

It is possible to transform sewage into these harmless products by direct oxidation, through the agency of the requisite organisms, in presence of air.

Thus, if a sample of sewage be shaken in a bottle with an excess of water saturated with air, and allowed to stand a sufficient time, under conditions which allow of an excess of oxygen being always present, it will be gradually transformed, and eventually nothing will be left in the bottle but a solution of the above salts, with some brown particles of 'humus,' and some carbon dioxide in solution.

Although it can be shown by careful analysis that the sewage suffers a regular sequence of changes, yet at no point are offensive gases evolved under these conditions—and neither marsh gas nor hydrogen is produced.

In the above case the sewage is purified under strictly *aerobic* conditions. In practice such conditions are met with when sewage is discharged into a stream or body of water, of such a volume that an excess of dissolved oxygen is always present, over that necessary to oxidise the sewage.

But it is rare to find conditions under which it is possible to deal with sewage in this way, by what may be termed the *dilution* method. A favourably situated outfall must admit

[1] Strictly speaking the term 'humus' should be reserved for organic residual matter of special chemical characteristics. For the sake of brevity the term is used here to include organic matters of somewhat indefinite composition which remain undecomposed at the end of the ordinary processes of purification of sewage. They are generally associated with a fair proportion of mineral matter, especially phosphates, and lime, and alumina compounds.

of the sewage being quickly mixed with a large excess of water, so that at all times the oxygen content is maintained at such a point that offensive products cannot be produced.

The extensive investigations of Letts and Adeney on the pollution of estuaries and tidal waters have resulted in the suggestion of various standards, in relation to the amount of oxygen available in the mixed sewage and tidal water, to meet the different conditions of discharge.

In the majority of cases, even where the dilution method is resorted to, and still more when it is a question of purification by application to land or artificial filter beds, some form of *tank treatment* is required, and the chemistry of this process may now be considered.

TANK TREATMENT OF SEWAGE

When sewage passes through a tank of any description, deposition of the heavier matters present will take place with greater or less completeness according to the method of construction of the tank, the rate at which the sewage passes through and the addition, or otherwise, of chemical coagulants to facilitate the deposition of the matters in emulsion.

We may consider tank treatment, according as it is directed, to effect one or other of the following results :—

(*a*) Simple sedimentation ;
(*b*) Anaerobic decomposition ;
(*c*) Aerobic decomposition ;
(*d*) Chemical clarification.

(*a*) **Simple Sedimentation.**—In this case we shall expect only the heavier matters in the sewage to be deposited, that is, the mineral substances, sand and silt, etc., together with paper, fæces, grease and soap. The character of the effluent will depend on the dilution or strength of the original sewage, and the distance between the sewage works and the source of the

sewage. The passage of sewage through sewers results in the mechanical breaking up and emulsification of fæcal matter, and the setting up of ammoniacal fermentation of the urea present in the urine. The extent to which these changes take place depends on the length of sewer to be traversed, and also on the state and construction of the sewer. New sewers with smooth surfaces will not readily allow the formation of deposits of sewage matter, and consequent further fermentative change. With well-laid sewers, only the initial stages of fermentation of nitrogenous matter will have set in by the time the sewage reaches the works, and such sewage should therefore be comparatively inoffensive. Moreover, in designing tanks for simple sedimentation, they should be of such a size that the sewage will not remain in them sufficiently long for any but the preliminary stages of decomposition to take place.

The *sludge* or deposit from such a sedimentation tank will, in consequence of what has been said, also need to be very frequently removed, if offensive decomposition is not to take place, and not only must it be quickly removed, but it must for the same reason be quickly disposed of, e.g., by trenching into the ground.

(*b*) **Anaerobic Decomposition.**—A tank designed to facilitate anaerobic decomposition differs from a sedimentation tank chiefly in being relatively larger, and so allowing time for decomposition to take place under anaerobic conditions. Such a tank has been variously termed a cesspool, a septic tank, a liquefying tank, or a hydrolytic tank. The differences in design are mainly structural, to facilitate deposition and removal of the solid matters, and to control more or less the character and extent of the chemical changes taking place. These chemical changes may at this point be usefully considered in detail.

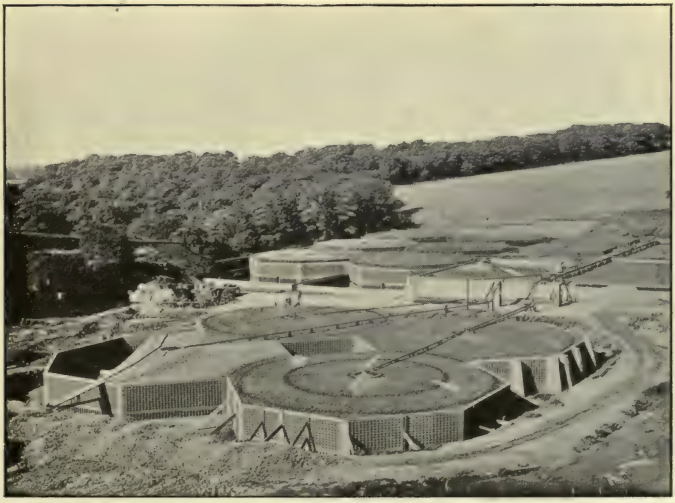
Decomposition of cellulose.—The anaerobic decomposition of cellulose has been considered in Chapter X. It was there

PLATE IV.



[*Photo by Author.*

(i) Sewage Works at Matunga, near Bombay.



[*Photo lent by W. J. Newton, A.M.I.C.E.*

(ii) Percolating Filters at Accrington.

shown to be due to two organisms, one of which produced mainly hydrogen and the other marsh gas. In both cases carbon dioxide (CO_2) and fatty acids are also produced as by-products. The production of gases is a visible indication that fermentation is taking place in the sewage. The evolution of *nitrogen* has often been regarded as taking place in septic tanks; the author's experience, however, would tend to show that such nitrogen, if it is produced, arises either from the air dissolved in the incoming sewage, or from the reduction of nitrates present therein, and not from the anaerobic decomposition of nitrogenous matter. It may be taken, therefore, that the gases which are evolved in the septic tank arise chiefly from the decomposition of cellulose. The researches of Omelianski, described in Chapter X, showed that the optimum temperature for this fermentation was above 90° F. For this reason the activity of septic tanks in this country, measured solely by the gas evolved, is much greater in summer than in winter, and it never attains the intensity observable in tropical countries. There, where the temperature seldom is less than 70° F. and often of course much higher, a quite extraordinary development of gas may take place. The illustration on Plate IV (i) is from a photograph taken by the author at the installation attached to the leper colony at Matunga near Bombay. Here the tanks are provided with gas-tight iron covers, and the gas is withdrawn from below these into a gas-holder. The carbon dioxide is removed by lime purifiers, and the inflammable marsh gas and hydrogen used for driving the engine which pumps the sewage, and also for lighting and cooking purposes. The gas-holder, lime purifiers, and engine-house are indicated in the photograph.

Such economic use of the gas from septic tanks has been, to a limited extent, adopted in this country, but, owing to the temperature conditions, it is hardly likely to find wide application on the large scale, and artificial raising of the temperature of large volumes of sewage is out of the question. It is,

however, worthy of suggestion whether in the case of small manufacturing premises, where much waste cellulose matter accumulates, e.g., packing paper, extracted plants from drug manufactories, waste hops from breweries, etc., it might not be economical to produce gas in this way in small tanks, maintained at the optimum temperature by waste steam.

Ammoniacal fermentation.—As already mentioned, the greater part of the nitrogen of sewage is present as urea, and the ammoniacal fermentation readily sets in ; in many cases indeed it may be almost complete before the sewage reaches the purification works. A great deal of discussion as to the necessity or otherwise of preliminary anaerobic treatment of sewage, has been confused by the failure clearly to distinguish between absolutely fresh sewage and sewage which has passed, it may be, through several miles of sewers. The consideration of the necessary conditions for nitrification, which found its place in Chapter XIII, shows the necessity for a preliminary conversion of urea, and allied substances such as amino acids, into ammonia before nitrification takes place. The author has found beyond question that if absolutely fresh sewage is to be put upon a filter, considerably more filter space is requisite to convert the nitrogen into nitrates, than if time is first allowed, e.g., by retention in tanks, for ammoniacal fermentation to take place. It is, however, rarely, in the case of an ordinary town sewage works, that sewage is met with in such a fresh condition ; as already stated, ammoniacal fermentation nearly completes itself in the sewers, or at any rate during a comparatively short tank treatment. It is doubtful whether urea would ever be found in an ordinary sample of town sewage.

Apart from the urea, however, the other nitrogenous constituents of the sewage have to be considered ; these are of a very complex character. Broadly speaking, all the various decomposition products of albumin will be represented in some form or other, together with actual undigested portions

of nitrogenous food. Apart from undigested food, it has been shown that the bulk of fæces consists of intestinal secretions, epithelium detritus, etc., and masses of bacteria. Under the conditions maintained within the anaerobic tank these will all gradually break down, and an important consideration here arises, viz., as to how far this breaking down should be carried. We have seen that eventually, by the decomposition of albuminous matter, evil-smelling substances such as hydrogen sulphide, indol, skatol, and various amines are produced. A frequent error in the design of septic tanks has been to make these too large, so that the decomposition of nitrogenous matter is carried more or less to its limit, with the production, in many cases, of serious nuisance. The design of anaerobic tanks should be directed to the rapid deposition of solid matter, and its retention for a period sufficiently long to enable it to be broken down as far as is economically possible, while the liquid portion of the sewage should be led away quickly, sufficient time only being allowed for ammoniacal fermentation and incipient proteolysis to take place therein. The hydrolytic tank of Travis, and the Emscher-Brunnen of Imhoff, have this object in view, but any design which distinguishes between the changes taking place in the solid matter and in the supernatant liquid is likely to be more or less successful. It has been found, e.g., that in latrine-tanks, where the greater part of the fæcal matter is retained in a compartment at the inlet, separated from the main tank by a pigeon-holed wall, that a very large amount of liquefaction of retained solids takes place in this inlet chamber.

Decomposition of fats.—There is evidence that besides the decomposition of cellulose and nitrogenous products a considerable change takes place in the fatty constituents of the sewage in the anaerobic tank. Fat is always present in household refuse from the washing of plates and dishes; all the soap which is used finds its way into the sewage, and

partially digested fatty matter is often present in fæces. Free fat is readily broken up by bacterial or enzyme action, yielding fatty acids and glycerine, as has been shown in Chapter XI. The higher fatty acids thus produced may be further broken down into soluble fatty acids of lower molecular weight. Soaps also are capable of change, but only very slowly.

In the case of small installations, attached to institutions such as sanatoria, asylums, etc., where a separate laundry exists, the author's experience strongly favours the separate retention of the soap, by treatment with lime salts, and collection of the precipitated lime soaps in specially devised intercepting traps. In such cases also it is desirable to retain the grease waste from the kitchen, which is quite capable of being readily and economically worked up into soap on the spot. The retention of fats, apart from its economic aspect, greatly simplifies the operations on the sewage works, where insoluble soaps are liable to be formed, causing accumulations in the tanks and clogging of the filters which receive the tank effluent. The decomposition of fat also gives rise to an extremely objectionable rancid odour, due to the formation of butyric acid.

To summarise, therefore, the changes which take place in the anaerobic tank, these are mainly the decomposition and gasification of cellulose, the ammoniacal fermentation of urea, the breaking down to a greater or less extent of more complex nitrogenous substances, and the splitting of fats. These changes are almost wholly due to bacteria and to enzymes, the latter in all probability present in fæces. The changes can be followed by analysis of the sewage and of the deposited sludge. In the liquid sewage it will be found that the free ammonia increases at the expense of the albuminoid; the oxygen absorbed from permanganate, while possibly not greatly differing at the beginning and end of the process in its total amount, will be found to be distributed in different

proportion between the easily oxidised matter and the more difficultly oxidisable substances, or, in other words, while the oxygen absorbed in four hours by the so-called ' four hours' test ' may not greatly decrease, the oxygen absorbed in three minutes will probably increase.

Whether there is an increase or decrease in *colloidal matter* depends upon circumstances. With highly concentrated sewages such, e.g., as are met with under conditions of very limited water supply, actual solution of colloidal matter undoubtedly takes place, owing to the breaking down, e.g., of albuminoid substances into amino acids ; on the other hand, with ordinary town sewage, it may readily be the case that the colloidal substances in the effluent from the tank increase, owing to the washing out of colloids from the sludge present in the tank. In considering the changes taking place in anaerobic tanks, the exact conditions of the installation must always be carefully borne in mind.

It was at one time considered essential that anaerobic tanks should be closed, in order to prevent access of light and air ; numerous experiments have shown that this condition is not necessary. It is obvious that beneath the immediate surface of the sewage in the tank the conditions must be anaerobic, and the covering of tanks is only called for by reasons of sightliness, or to render more permanent the scum, which generally forms owing to the throwing up of solid matter by the gases evolved during the fermentation of the sludge. A cover, of course, is necessary if these gases are to be collected and utilised as in the case quoted, but is of little use for the prevention of nuisance unless escaping gases are collected and passed through a washing tower, e.g., of coke, down which a spray of water passes.

(*c*) **Aerobic Decomposition.**—In the early experiments of Mr. Dibdin at Sutton in Surrey, during the later nineties, the attempt was made directly to treat crude sewage on a coarse

contact bed, that is, a tank filled with large pieces of coke, burnt clay or other material. Such a tank is first filled up with the sewage, when the insoluble and colloidal matters are, for the most part, deposited upon the surfaces of the pieces of material in the tank, and the liquid allowed to run off from the bottom of the tank. Air enters the interstices of the medium to replace the liquid, the tank is allowed to remain empty for some hours, and opportunity is afforded for the deposited organic matter to be oxidised; the tank is then filled again with sewage and the cycle of operations repeated. A tank of this kind may be termed an aerobic tank, and the changes which go on in it are essentially different from those taking place under anaerobic conditions, as described under (*b*).

The process used at Sutton acted well in so far that the heavier suspended matter in the sewage was largely removed, and converted in course of time into a nearly odourless residuum. The main drawback to the process was the gradual blockage of the interstices of the medium and the difficulty of cleaning it without complete removal from the tank. This difficulty Dibdin seeks to avoid in his recently introduced slate bed. In this case, instead of the tank being filled with irregular lumps of material, superimposed horizontal layers of slate are made use of, separated by distance pieces about two inches thick. On filling the tank with sewage, the suspended solids deposit themselves on the slates, and are gradually oxidised in the same manner as in the Sutton process. It is possible to remove the deposit from time to time from the surface of the slates by flushing out, and so to retain the water-holding capacity of the tank undiminished. The writer has had occasion to examine with some care the changes which go on in these slate beds, as they are called. He found that the oxidation of the organic matter, and especially of the fatty constituents, is largely due to masses of nematode worms, with infusoria, etc., and, of course, bacteria. The deposit on the slates, in course of time, assumes a liver-like

consistency and can be stripped off in pieces and examined; the smell is not offensive, being similar to that of an exposed river bank.

If some of the material is placed in a glass tube and air led over it, considerable volumes of CO_2 are given off, through the respiration and other changes of the organisms present. If the deposit is covered with water and air is excluded, it very soon putrefies and becomes offensive; it is evident, therefore, that in the working of such tanks care must be taken that the conditions at no time become anaerobic. When the sewage first enters the tank a considerable amount of air is dissolved in it, as it falls through the slates, and a further quantity is trapped underneath the slate surfaces; this is sufficient to maintain aerobic conditions for an hour or two, which is the length of time which should be allowed to elapse before the tank is emptied. On flushing out the deposit and allowing it to drain and weather in the air, it is gradually converted into a brown inoffensive mass, resembling garden mould.

(*d*) **Chemical Clarification.**—In the chapter on the chemistry of albumins it was shown that colloidal substances of this nature could be coagulated and precipitated by addition to their solutions of hydrated precipitates, such as those of iron and aluminium hydroxides. This precipitation is made use of for the clarification of sewage. The chief precipitants used are aluminium sulphate, ferric sulphate, lime and ferrous sulphate (green copperas) used in conjunction. The choice of precipitant depends on the relative market price of the particular chemicals, and on the facilities available for their efficient use.

The right adjustment, e.g., of lime and copperas so as to keep the lime always slightly in excess, requires constant attention, whereas salts of alumina are precipitated directly by the carbonate of ammonia present in the sewage. Ferric

salts have been found to be specially useful in the case of sewages containing an excessive amount of grease, e.g., at Wakefield, where much wool-scouring refuse enters the sewers.

All processes of chemical precipitation, while they are capable of yielding effluents containing less suspended matter than either of the processes considered in the foregoing paragraphs, result in the production of considerable quantities of sludge, which needs special care in its disposal, as its constituents are still capable of undergoing offensive decomposition, differing thus from the residuum left after well-conducted anaerobic or aerobic treatment.

The choice of one or the other of the methods of tank treatment (*a*), (*b*), (*c*) or (*d*) depends on local conditions. In the case of small communities, where constant attention cannot be given, and also where the fall is limited, anaerobic tanks find useful application. In certain cases also, notably at Birmingham and to some extent at Manchester, anaerobic treatment has been found useful, in the first case in order to produce an inoffensive sludge, and in the second case to neutralise to some extent the effect of antiseptic trade effluents present in the sewage, before the latter is finally treated on filter beds. In both these cases, however, the presence of considerable quantities of iron salts in the sewage diminishes the chance of nuisance, owing to the combination of any sulphuretted hydrogen produced with the dissolved iron, to form black ferrous sulphide. It must be emphasised that anaerobic treatment, carried out in ill-designed tanks and with imperfect supervision, may be, and often has been, a serious source of nuisance, and, for this reason, preliminary aerobic treatment has often much to recommend it. The slate process of Dibdin requires, however, an amount of fall depending on the depth of the bed, in addition to that required for the subsequent filtration processes. If this is available, the process can often find useful application, it being understood

that some form of catchment tank is necessary to retain the suspended matter coming away from the slates.

It was at one time thought that the clarification of sewage by means of chemicals must give place entirely to biological treatment of one sort or another. The findings of the Royal Commission on Sewage Disposal have, quite rightly, in the author's opinion, suggested that many cases still exist where this method of purification is to be preferred. Where the large amount of sludge produced by chemical precipitation can be easily and cheaply disposed of, and where the space available for the final filtration process is limited, the total expense involved will probably be less by this method than by any other, owing to the longer life of the filter beds in consequence of the small amount of suspended solids passing on to them. A typical case for the application of chemicals is afforded by the conditions of the sewage works at Salford. Here the available area of filters is necessarily very restricted, owing to the site of the works, and to maintain the high rate of filtration necessary if the sewage is to be dealt with thoroughly, preliminary treatment is called for. On the other hand, as the sludge is sent to sea in a steamer, the standing charges of which have always to be maintained, an increase in the sludge production does not necessarily mean a proportional increase in the cost.

There are definite limits to the economic use of chemicals. It has been shown that beyond a certain point an increase in the amount of chemicals added does not produce a proportional reduction in the amount of suspended matter. Further, with very dilute sewages, the colloidal matter to be removed is disseminated through a large volume of water, so that very considerable quantities of chemicals have to be added in order to precipitate it, and here again the cost is out of proportion to the purifying effect obtained. Speaking generally, therefore, the use of chemical clarification may be recommended where the sewage is concentrated, where the

available filtration area is limited, and where the sludge is easily disposed of.

A further case for chemical treatment may arise where special trade effluents are present. Thus, at Bilston and Wolverhampton, large quantities of lime have to be added, to neutralise and precipitate the acid solution of ferrous chloride, or 'iron pickle,' discharged into the sewers from galvanising works.

THE FINAL PURIFICATION OF SEWAGE

In general, as has been explained, some form of preliminary treatment is necessary before sewage can be finally mineralised in biological filter beds. It is possible, however, under special conditions to treat crude sewage directly on filters. Where the sewage is dilute, and where considerable fall is available, the liquid, after efficient screening and removal of the coarser solids, sand, etc., in catchpits, may be directly sprayed upon coarse percolating filters of considerable depth. In this case the oxidation of the suspended and colloidal matters takes place by much the same agencies as are at work in the slate bed, above described, and the resulting granular residue passes out at the bottom of the filter, and can be retained in catchpits, or on the surface of sand strainers. The works at Rothwell in the West Riding of Yorkshire have been successfully designed on these lines. The conditions differ in such a filter from those obtaining in the slate bed, in that the liquid portion of the sewage passes in a thin film over the filtering medium, and its soluble impurities are therefore oxidised as well as the matters in suspension. If the rate of filtration is not too high, it is even possible completely to oxidise fairly strong sewage in this way. A periodical renewal of a portion of the filtering medium is, however, likely to be called for in such a case. A good instance of the adequate treatment of strong sewage is to be seen at Little Drayton, where a filter,

on the plan devised by the late Colonel Ducat, has been in use for many years, and has been reported upon by the Royal Commission.

In the majority of cases it will probably be necessary, or at any rate preferable, to adopt some form of preliminary treatment for the sewage, before its final purification on filter beds, and we may now consider the changes which take place when such partially treated sewage is applied to filters.

The artificial filters in general use are of two types, which may be broadly divided, according as the sewage is applied intermittently or continuously, into—

(*a*) Contact beds;

(*b*) Percolating or trickling filters.

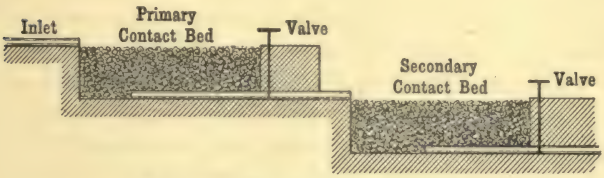


FIG. 27.—CONTACT FILTER BED.

(*a*) **Contact Beds.**—The general design of a contact bed is seen in Fig. 27. It consists of a water-tight tank, generally of concrete, filled with filtering material carefully screened and graded to a definite size. The essentials of such a material are that it should be durable, that is, not likely to crumble on use, and should expose as large a surface as possible. Hard well-fused clinkers fulfil this condition most perfectly, but other materials may also be used, if good clinker is not available. Effluent from the preliminary process is passed on to such a bed, and allowed to remain in contact, generally for about two hours, and then run off; and if not sufficiently purified, submitted to similar treatment on another bed, at a lower level. The material in the second contact

bed must be of smaller dimensions than that in the primary bed, if the best results are to be obtained. In special cases a third treatment on still finer grade material, e.g., sand, may be called for.

The following are the principal changes which take place in a contact bed. The suspended and colloidal matter, still present in the liquid to be treated, is mechanically retained by the filtering medium. It is evident, therefore, that the fineness of this medium must increase, as the amount of suspended and colloidal matter decreases, if this mechanical effect is to be obtained. Besides the mere mechanical straining, a species of absorptive action also takes place between the surface of the medium and the constituents of the sewage, which increases within limits, as the slimy layer thus formed on each fragment of filtering material becomes more well defined. This slimy layer also acts as a sponge retaining an appreciable proportion of the liquid applied, together with its dissolved constituents. A considerable amount of purification will therefore take place by purely mechanical and absorptive action, immediately the liquid is applied to the filter. This action, however, would very soon cease, and the contact bed become clogged and foul, were it not for the biological activities which are set up within it. These activities are exceedingly various, and depend not only on the life of bacteria, but on many higher organisms, notably small worms and many species of infusoria. Recent researches, carried out more particularly at the Government Experimental Station in Berlin, have emphasised the functions of these higher organisms, and it is here that the choice of the preliminary treatment, whether by simple sedimentation, by anaerobic or aerobic tanks, or by chemical precipitation, needs careful study. If a sample of sewage be collected in a sterile bottle, and allowed to stand freely exposed to the air, but protected from infection by a plug of cotton wool, a film of organic life generally makes its appearance. If this is carefully examined under the

microscope, after the lapse of some days, or even weeks, numerous forms of life are generally visible. This life, potentially present in the sewage, is probably an important initial source of population of the sewage filter beds. The effect of the different methods of preliminary treatment, above referred to, upon this organic life, has been only imperfectly studied as yet. We should expect, *a priori*, that effluents from simple sedimentation, or from the aerobic tank, would be more favourable to the existence of aerobic organisms of this sort than either anaerobic treatment, which might destroy them owing to the absence of oxygen, or chemical precipitation, which would tend mechanically to remove them. It is not unlikely that the organic life of sewage will vary according to the amount of subsoil and surface water drainage entering the sewers. The author has indeed found, in investigating the conditions of purification of sewage obtained in an absolutely fresh condition, without admixture of surface water, that decomposition and nitrification take place with extreme slowness, when the sewage is allowed spontaneously to oxidise in a bottle. Inoculation, by means of medium from a filter, greatly accelerated the rate of oxidation. He has further found that the effluent from an aerobic tank oxidised spontaneously more quickly than the effluent from chemical precipitation, containing an equivalent amount of oxidisable matter.

Whatever the primary source of the population of a sewage filter bed may be, whether derived from the original sewage or from the bacteria naturally present in all unsterilised material, such as is likely to be used for the construction of such filters, there is no doubt that, in course of time, countless numbers of bacteria, and other organisms of the nature specified above, establish themselves in the filter. During the period when the contact bed is empty, and when consequently its interstitial spaces are full of air, these organisms act upon the suspended and dissolved impurities retained

by the filtering medium. Unbroken down albuminoid matter is further peptonised, and ammonia is oxidised to nitrite and finally nitrate. Fatty acids and other carbonaceous matters are finally oxidised to CO_2. This can be verified, if the gases in the interior of such a bed are drawn off and analysed, when a marked increase in the CO_2 over that present in the atmosphere will be noticed. Moreover, if a portion of the filtering material is carefully removed from the bed without disturbing its coating of slime and is placed in a closed vessel provided with a manometer, an appreciable rise in the mercury may be observed, owing to the absorption of the oxygen in the containing vessel. The presence of nitrates can be determined by washing the material with water free from nitrate, and testing for the presence of the latter in the washings.

The changes just described take place while the bed is standing empty, and are characterised by the predominance of nitrification; when the bed is again filled with liquid a somewhat different set of conditions arises. Mechanical absorption of the more insoluble matter takes place as already described, but oxidation also occurs, through interaction of the nitrates present with these substances and with impurities present in solution; in this way finely divided cellulose may be finally oxidised, as was explained in Chapter X. During these changes, which may be grouped together as *de-nitrification* changes, loss of nitrogen occurs, as has been shown in Chapter XIII, either as free nitrogen or, it may be, as nitrous oxide, N_2O, this gas having actually been discovered by Letts in solution in the liquid contents of a contact bed.

The proper working of a contact bed can be controlled, by having regard particularly to the amount of nitrate present in the effluent, especially in the first discharge after a long period of rest. The nitrates present represent the overplus left after de-nitrification has taken place; within limits, the longer the

period of standing empty, the greater will be the amount of nitrate found, but if nitrates are present at all, it is evident that the conditions are still mainly aerobic, and therefore suited to the maintenance of organic life. If a contact bed becomes clogged and waterlogged, not only will nitrates be absent in the effluent, but very often crowds of worms will emerge at the surface of the bed, seeking their necessary air supply.

An interesting application of de-nitrification has been made by Letts at Belfast, whose object was to produce an effluent containing as little nitrogen as possible, either in the form of ammonia or nitrate, in order to minimise the growth of *Ulva latissima*, which was found to derive its nitrogen equally well from either of these sources. Letts purified a portion of his effluent by means of trickling filters in order to obtain as high a yield of nitrate as possible; this nitrified effluent was then mixed with the remainder of the unfiltered effluent, and the mixture treated on a *de-nitrifying* bed. The nitrates in the one portion interacted with the organic matter in the other, with elimination of nitrogen, and production of a purified effluent, containing a minimum of nutriment for the *Ulva*.

(*b*) **Trickling or Percolating Filters.**—The operation of a trickling filter differs from that of a contact bed, in that the liquid is applied to it in such a way that it flows over the fragments of filtering medium in a thin film, and the oxidation process is consequently continuously proceeding. It is in this sense that the trickling filter may be spoken of as a continuous filter, as distinguished from a contact bed, whose operation is intermittent, and clearly divisible, as we have seen, into two distinct processes. Mechanically speaking, it is doubtful whether a really continuous filter has yet been constructed. When a sewage effluent, e.g., is sprinkled upon a trickling filter by an ordinary rotary distributor, the operation is really, of course, a discontinuous one, each element of surface

receiving a dose of liquid at given intervals of time, depending on the speed of rotation of the sprinkler.

It is not necessary here to describe in detail the various methods for applying sewage effluent to trickling filters, an account of them will be found in text-books dealing with the engineering side of the problem. It will only be briefly mentioned that distribution may be effected by simple intermittent discharge on to a surface of fine material, by rotary distributors such as are indicated in Plate IV (ii), by spray jets (Fig. 28), and by other mechanical devices of more or less complexity.

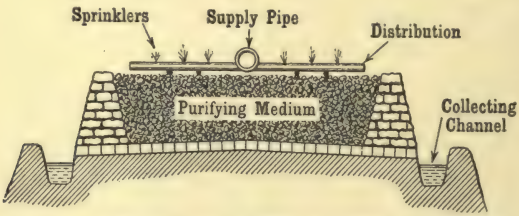


FIG. 28.—PERCOLATING FILTER WITH SPRINKLERS.

Plate IV (ii) shows a set of trickling filters at the Accrington Sewage Works, which will sufficiently indicate their general appearance.

We have here carefully to consider, assuming equable distribution of the liquid upon the filtering material, what the physical and mechanical conditions are which result in the production of a purified effluent.

The efficiency of a sewage filter depends on the total effective surface area of the filtering material, together with a sufficient air supply. By effective surface area is here meant the sum of the surface areas of the fragments of material. The surface area of the filter may be spoken of as the upper surface area. Thus, if large-sized material is used, a greater quantity of it is necessary in order to obtain the same total

surface area. On the other hand, the material may be so far subdivided that the interstices rapidly fill up with gelatinous matter, which in its turn holds up water, so that the interstices become reduced and the circulation of air is interfered with.

In general, therefore, it will be found economical to use the smallest material which allows of free circulation of air. The main direction of air circulation in a trickling filter is probably from above downwards, the air being drawn through the filter by the percolation of the liquid. An exception to this rule may occur in cold weather, when the higher temperature of the filter, as compared with the outside air, may induce an upward current.

It is obvious that filtering material must be avoided which tends to weather and break down, as the interstices will then tend to be filled with small pieces of broken-down material, and air circulation will be impeded. In order to obtain the greatest possible surface area, material of an irregular character, such as hard furnace clinker, gives the best results, but other available material can be used, so long as it is not so smooth as to exert little or no retaining power, or retentivity, on the gelatinous matter deposited upon it.

Further, thorough drainage is essential; otherwise water will tend to be held up by capillary attraction in the bottom layers of the filter, and will interfere with air circulation. For this reason a concrete bottom for the filtering medium is advisable, the thickness of which will depend on engineering considerations. These conditions of efficiency apply equally both to contact beds and to trickling filters.

It is obvious that if the filtering material is to be fully made use of, efficient and equable distribution of the effluent over every part of the filter is essential; it may, however, be pointed out that in certain circumstances, especially in small works, it is well to have an ample surface area of material, so that the efficiency of the process shall not be too dependent on

the exact operation of mechanical devices; in other words, a large factor of safety should be provided.

The physical conditions governing the rate of passage of liquid through trickling filters have been studied by W. Clifford in the researches referred to on p. 227.

We are now in a position to follow the changes which take place in such a filter. In the first place, as in the contact bed, a purely mechanical effect is exerted, and the suspended and colloidal matters deposit themselves on the surface of the medium. This will take place obviously to a greater extent in the upper layers of the filter, and there is consequently a limit to the depth of such filters, owing to the concentration of deposited matter in the upper layers, which will take place if such effluent is poured upon them at a very high rate. For this reason also, trickling filters are better adapted to deal with large volumes of dilute effluent, rather than with a more concentrated liquid, the application of which results in a rapid accumulation of undigested organic matter in the upper layers of the filter. In course of time forms of life establish themselves in these filters, worms, larvæ, infusoria and bacteria, which maintain the cycle of changes. Albuminoid substances are broken down to amino compounds, and finally oxidised to nitrates. The trickling filter differs from the contact bed primarily in the predominance of nitrification, owing to the constant presence of oxygen in its interstices. No doubt some de-nitrification takes place in the interstices of the medium, but speaking generally, a greater proportion of the nitrogen is recovered as nitrate than in the case of a contact bed. A further great advantage possessed by the trickling filter is that the effluent passing away from it is constantly saturated with dissolved oxygen, and consequently the effluents from these filters contain in general a greater reserve of oxygen, available for further purification in the stream into which the effluent may flow. On the other hand, owing to their method of operation, there

is a greater tendency for incompletely oxidised nitrogenous matter to pass away from them, either in solution, in the colloidal state, as granular residual ' humus,' or as débris of growths formed in the filter. In the contact bed, as has been shown, the oxygen of the nitrate interacts with the undecomposed oxidisable matter during the period of standing full.

It is necessary, in the case of trickling filters, that means should in all cases be provided for arresting suspended matter which continuously passes away from them. For this purpose either so-called ' humus tanks ' or sand strainers may be employed. Especially are these necessary after the filter has had a period of rest. The colloidal matter deposited on the filtering medium suffers oxidation during such times, and is rendered granular, and readily detaches itself in consequence from the filtering material. At such times, therefore, the effluent will contain abnormal quantities of solid matter. This is also the case in spring time, when the organic life in the filter is particularly active. The material which has been stored during the previous months is then to a large extent ejected from the filter.

This phenomenon is further instructive, as showing that the changes taking place in these filters are by no means instantaneous, but take place over a prolonged period of time.

CONDITIONS GOVERNING CHOICE OF FILTRATION METHODS

In designing works for the purification of sewage, the choice of method for the final purification of the effluent must depend on a number of factors, more particularly the following: (*a*) area of land and fall available; (*b*) strength of sewage; (*c*) methods of preliminary treatment. The full discussion of this aspect of the question involves engineering considerations, which are outside the scope of this work, but it is obvious that deep trickling filters can only be made use of economically when there is sufficient fall between the

outlet of the tanks and the point of final discharge. Moreover, even if the total amount of fall be adequate, yet if the ground slopes gradually away, it may be more economical to put down primary and secondary contact beds and so avoid the excavation necessary for deep trickling filters. The amount of solid matter discharged from contact beds, especially from secondary beds of fine material, is not so great as from open percolating filters, and consequently, in such a case, the final provision of humus tanks may be on a less extensive scale. This is sometimes a factor in the choice of method. On the other hand, the material in contact beds generally needs to be removed and washed more often than the medium in trickling filters.

As already indicated, a concentrated sewage lends itself for various reasons to treatment by contact beds, at any rate as a preliminary step. Probably the most satisfactory method in such a case is preliminary treatment in contact beds, followed by final purification on trickling filters. For weak sewages, it is probable that trickling filters are always to be preferred. The method of preliminary treatment conditions primarily the grade of material to be used on the filter bed. The freer the effluent from suspended or colloidal matter, the finer the grade of material that can be economically used. Where there is little depth available for filter beds, the thorough clarification of the sewage may be desirable, so that fine-grade material can be used, and the lack of depth made up for by the extended surface area of the particles.

THE INTERPRETATION OF SEWAGE AND WATER ANALYSES

The methods used in the analysis of sewage and sewage effluents must be looked for in the books specially devoted to the subject. A short space may be usefully given here to the interpretation to be placed on the results of these analyses, when viewed in the light of the information given in the foregoing pages.

The methods of sewage and of water analysis are closely allied, the chief difference being in matters of detail, necessitated by the different quantities of oxidisable or organic matter which have to be determined in a given volume of the respective liquids. As a matter of fact, a sewage effluent of high quality may contain no more organic matter than a low-grade drinking water.

The main difference between the two branches of analysis lies in the significance of the presence of nitrate in the two cases, and the importance attaching to the determination of the number and character of the bacteria present.

A good sewage effluent, as we have seen, is generally characterised by the presence of an abundant proportion of *nitrates*. The presence of nitrates in a water supply may often, on the other hand, give rise to suspicion, as pointing to the oxidation of previously present organic matter. An exception to this rule is met with in the case of deep well waters, where the nitrates may arise from long past deposits. In such cases, as a rule, the nitrates will be unaccompanied by *nitrites*; the presence of the latter, which are unstable intermediate substances, point to an oxidation process in actual operation, or possibly, of course, de-nitrification changes which may be equally due to organic matter.

In regard to the presence of *bacteria*, these are of comparatively little significance in the case of an ordinary sewage effluent, as none of the processes of sewage purification in common use, short of sterilisation or slow sand filtration, do more than reduce the number of organisms present. For this reason the detection of *Bacillus coli* in a drinking water is presumptive evidence of sewage pollution. This test is one of extreme delicacy and it is, therefore, quite possible for a sample of water to pass the usual chemical tests, and yet to be placed under suspicion, when examined bacteriologically.

The Analysis of Sewage.—Bearing in mind the importance

of chemical evidence in the case of sewage or sewage effluents, the factors generally determined in a sewage analysis are :—

(*a*) Total *oxidisable matter* as measured by the oxygen absorbed from acid permanganate in four hours and in three minutes ;

(*b*) *Nitrogen*, either ammoniacal, albuminoid, nitrous or nitric ;

(*c*) *Chlorine ;*

(*d*) *Suspended matter ;*

(*e*) *Putrescibility ;*

(*f*) Consumption of *dissolved oxygen.*

The objects of sewage analysis may be defined as follows : —

(1) To determine the character of the sewage to be treated.

(2) To determine the efficiency of purification works.

(3) To determine the effect of the discharge of sewage or effluents into various bodies of water, either river, lake or sea.

1. Taking these objects in order, it is of great importance, when designing works for sewage purification, to ascertain the concentration or *strength* of the sewage to be treated, as the amount of filter space provided must necessarily depend on the amount of organic matter to be transformed.

The Local Government Board has recently issued a memorandum, based on the Fifth Report of the Royal Commission on Sewage Disposal, which defines roughly what is meant by ' strong,' ' average,' or ' weak ' sewage. Using permanganate, 1 c.c. of which equals one milligram of oxygen (which is ten times the strength frequently used), and assuming that the determination is made at 80° F., the amount of oxygen absorbed by the different strengths of sewage is taken as follows :—

' Strong ' sewage 17 to 25 parts per 100,000
' Average ' sewage 10 to 12 parts per 100,000
' Weak ' sewage 7 to 8 parts per 100,000

The other analytical figures, in the absence of trade effluents, will probably vary in proportion.

It is, of course, necessary in determining the strength of sewage that an average be taken if possible over several days, samples being taken hourly and mixed in proportion to the rate of flow.

2. In determining the *efficiency of purification* works, the analysis will show the progressive reduction in impurity attained in the various stages of the process. The oxygen absorption, the ammoniacal and albuminoid nitrogen and the suspended matters should decrease. A considerable proportion of the nitrogen should reappear as nitrate. The resultant effluent should have lost its putrescibility, that is to say, when kept in a closed and full bottle for a few days, at a temperature, say, of 80° F., it should not become offensive.

The *chlorine* figure, which is due to the sodium chloride present in the sewage, is unaltered by the purification process, and therefore serves as a useful index to show whether the effluent really represents the sewage from which it is produced. In a true comparison the chlorine number should be the same in both cases.

3. The effect of an effluent upon a body of water depends essentially, as we have seen, on the amount of *dissolved oxygen* it is capable of abstracting from a body of water, and the Royal Commission have therefore summarised, as it were, the various methods of sewage analysis, and have sought to define a good effluent in terms of its power of consuming dissolved oxygen. The importance of the absence of suspended solids, which may form troublesome deposits, is also recognised, and they suggest that an effluent would generally be satisfactory if it complied with the following conditions :—

(1) ' That it should not contain more than three parts per 100,000 of suspended matter; and

(2) That after being filtered through filter paper it should not absorb more than :—

(*a*) 0·5 part by weight per 100,000 of dissolved or atmospheric oxygen in twenty-four hours ;

(*b*) 1·0 part by weight per 100,000 of dissolved or atmospheric oxygen in forty-eight hours ; or,

(*c*) 1·5 parts by weight per 100,000 of dissolved or atmospheric oxygen in 5 days.'

Although these tests are open to some criticism in matters of detail, they do broadly serve to determine whether an effluent is likely to give rise to nuisance or not. They may be hardly stringent enough for special cases, e.g., if the effluent enters a stream used for water supply ; or on the other hand may be unnecessarily severe, when ample dilution takes place, and the water into which the effluent is discharged is not used for drinking.

The adequacy or otherwise of the purification effected under given conditions can generally be judged from a careful examination of the conditions obtaining at the point of discharge, especially the various forms of living growth which can be there observed. The presence of *Beggiatoa*, for instance, would indicate that unoxidised sulphides are still present, and, consequently, that the purification was imperfect. Such a state of things is almost certain to give rise to nuisance. Other forms of sewage fungus, such as *Sphaerotilus natans* or *Leptomitus lacteus*, are also characteristic of imperfect purification. Certain protozoa, such as *carchesium*, indicate a more satisfactory purification, still stopping short, however, of complete mineralisation. A first-class effluent can generally be recognised by an increased development of healthy aquatic vegetation in its vicinity, owing, doubtless, to the nitrates present.

The Analysis of Water.—Turning now to the subject of the analysis of water, while it is true that water may contain

very little organic impurity—so that, on the results of chemical analysis alone, it might be passed as satisfactory, and yet reveal the presence of *B. coli* when examined bacteriologically —yet in the author's experience, if a series of comparative samples are taken, and the analysis carried out with special care, the chemical and bacteriological indications are usually of the same character, and the conditions which tend to improve the chemical composition of the water, tend also to the removal or diminution of dangerous organisms.

Thus, e.g., Houston has shown that prolonged storage tends gradually to decrease the number of organisms present in a water supply, and especially the less resistant organisms such as the typhoid bacillus. There is no doubt that the number of bacteria decreases as the amount of pabulum diminishes, and *vice versâ*. Recent experiments in France by Miquel and Mouchet have shown that the impurities in water can be oxidised by spraying over filters worked on similar principles to the sewage trickling filter, but of course with material of smaller dimensions. With the chemical improvement of the water, there is again diminution in its bacterial content, but an extraordinary increase in the number of organisms takes place if the filters are dosed with a solution of peptone.

Besides the gradual destruction of their pabulum which takes place on storage, the effect of sunlight is of great importance in diminishing the number of bacteria present, especially certain kinds, and those the more dangerous. This aspect of the matter has been dealt with by Major W. W. Clemesha, in his extensive study of the bacteriology of drinking water supplies in tropical countries, undertaken particularly in reference to the water supplies of Madras. He endeavoured in his researches to differentiate the various organisms allied to *Bacillus coli*, by an extension of the method suggested by MacConkey, who divided fæcal bacilli into four groups :—

Group I. Ferments neither saccharose nor dulcite;
Group II. Ferments dulcite but not saccharose;
Group III. Ferments dulcite and saccharose;
Group IV. Ferments saccharose but not dulcite.

To these Major Clemesha added sundry other fermentative tests, whereby he was able to some extent to classify numerous varieties of coli-like organisms present, all of which are capable of fermenting lactose. He found that certain of these were characteristic of water which was obviously recently polluted; others, on the other hand, alone survived when the water had been exposed to the sun for some time, or was drawn from a well after long drought, etc.

As nearly all the chief water supplies in tropical countries are, so to speak, of natural origin, that is, from wells, rivers or lakes, and are subject to occasional pollution, and, therefore, according to English standards would be classified as dangerous, it is obviously of importance to be able to differentiate between the residue of pollution and the presence of deleterious matter of recent introduction. While, no doubt, further research and many more data are requisite before it is possible, under all circumstances, to distinguish between harmless and potentially dangerous supplies by the characteristics of the organisms present, or by the chemical reactions which they produce under given conditions, Major Clemesha's researches are a very interesting application of bacteriological chemistry to the classification of water supplies.

The Biological Purification of Trade Effluents.—Numerous effluents from manufacturing processes are highly charged with organic matter, and are capable of bacteriological purification by methods analogous to those used in the purification of sewage; such effluents are, e.g., those from breweries and distilleries, from tanneries and hide-dressing works, from beetroot sugar factories, starch works, wool-scouring works, bone manure and glue factories.

Special methods have to be used in each case, according to the character of the effluent to be treated, and dilution is frequently necessary, e.g., in the case of pot ale from distilleries, before purification can be effected. In the case of effluents containing sugar or starch, care has to be taken lest acid fermentation should set in, especially formation of butyric acid, which is liable to create serious nuisance. For this reason it is generally found necessary to avoid anaerobic treatment in the case of these effluents, and a preliminary addition of lime is often advantageous.

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